RESULTS AND DISCUSSION

Minced fish flesh can be utilized directly to make fish sticks and portions, thermally processed as well as extruded fish products. On the other hand, frozen minced fish could be used also as cooking materials. The yield of mince with respet to whole fish reached 44.52 % for hake (*Merluccius merluccius* L.) and 56.67 % for sardine (*Sardine pilchardus* W.)

Part I: Chamical and physical propertias of minced fish during frozen storage:

* Chemical composition of minced fish:-

Variations in the chemical composition of the inves_ tigated minced fish samples; hake, sardine and the prepraed mixtures were followed during storage for 12 month at -20°C are seen in table (1).

Data presented in table (1) showed the level of moisture content during frozen storage of the tested lots A, B, C, D, E. The corresponding values of moisture were 82.48, 78.97, 75.37, 72.02, and 68.62 % respectively. Form the aformentioned results, the moisture content of lot A was relatively higher than that of the other lots of minces and lot E came in the later leve. However the available data proved the stability of moisture of the tested samples under the frozen storage condition used. These results are in agreement with Botta *et al.*(1983) who stated that moisture content was relatively constant throught frozen storage of non-spawning capelin.

With regard to the fat content of the sample results of (Table 1) show that lot (E) which is consdered to be among the fatty fish contained higher level of fat (11.95 % fresh weight). The low level of fat content was found in lot A (0.99 % on fresh weight) and so it could be

Table (1) Major chemical constituents of the investigated fish samples under frozen storage condition of -20°C for 12 months.

Moisture content (%)

Months	Lot A	Lot B	Lot C	Lot D	Lot E
0	82.48	78.97	75.37	72.o2	68.62
3	82.91	79.26	76.01	72.37	68.88
8	82.65	79.21	76.01	72.38	69.26
12	82.65	78.97	75.92	72.50	69.39

Protein content (%)

Months	Lot A	Lot B	Lot C	Lot D	Lot E
0	16.14	17.20	17.48	17.91	18.27
3	16.o6	16.88	17.84	17.80	18.42
8	16.19	16.67	17.19	17.95	18.29
12	16.14	16.66	18.03	19.09	19.14

Fat content (%)

Months	Lot A	Lot B	Lot C	Lot D	LotE
0	0.99	2.92	5.31	7.06	11.95
3	0.69	2.61	5.90	7.91	11.91
8	0.77	2.80	5.50	7.44	10.79
12	0.65	3.24	5.63	7.65	10.69

Ash content (%)

Months	Lot A	Lot B	Lot C	Lot D	Lot E
0	0.98	1.00	1.11	1.14	1.16
3	1.02	1.14	1.09	1.24	1.39
8	1.24	1.18	1.16	1.14	1.24
12	1.12	1.15	1.15	1.14	1.19

Lot A: 100 % hake, Lot B: 75 % hake + 25 % sardine
Lot C: 50 % hake + 50 % sardine
Lot D: 25 % hake + 75 % sardine, Lot E: 100 % sardine

considered among lean fish. Variations in fat content among lots B, C and D were markedly followed by the difference in the ratios of mixtures between the two species of minced fish. It is also found that slight decrease in the fat content was noticed in lot A and E at the end of storage period. While in the other lots B, C, and D fat percentage was nearly constant. Same finding were mentioned by Baaiu (1974) who showed that fat percentage of mackerel was nearly constant through frozen storage at -25°C for 6 months. However, the loss of fat content might be due to oxidation and hydrolysis of lipids which lead to the formation of some volatile compunds as aldehydes and ketons.

The aformentioned results are in agreement with those given by Careche and Tejada (1991) who showed that the pro_ximate analyses of minced fish flesh for hake were: crude protein (N x 6.25) 16.96 %, lipids (0.98 %), moisture (81.45 %) and ash (1.24 %). They also gave the following analysis sardine: crude protein (17.31 %) fat (12.62 %), moisture (67.79 %) and ash (1.23 %). Huidobro, *et al* (1995) gave the proximate analyses of minced sardine muscle to be: crude protein 17.8 %, crude fat 13.2 %, moisture 66.5 % and ash 1.4 %. This analysis of the main constituents is considered normal for a species of fatty fish and is similar to those published by Jiménez-Colmenero *et al* (1988) for samples of the same species taken at about the same time of year, and the proximate analyses of the hake muscle were similar throughout the year. Love (1988) stated that the amount of water and fat varies depending on species but were together about 80-85% of the total weight.

* **pH value:**

Variations in pH values during frozen storage at -20°C for 12 month of minces prepared from hake, sardine and the prepared mixtures are shown in figure 1. Analysis of

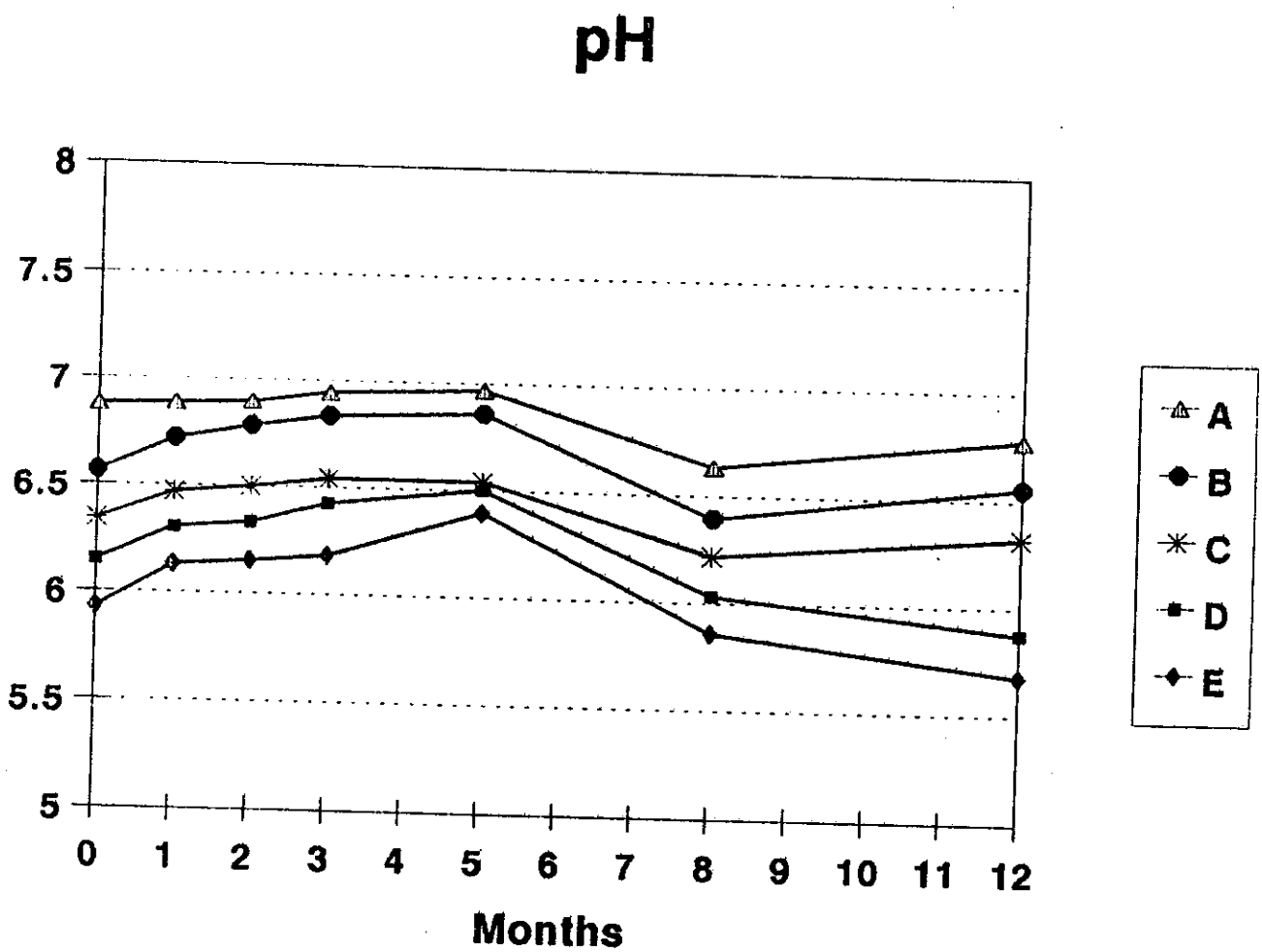
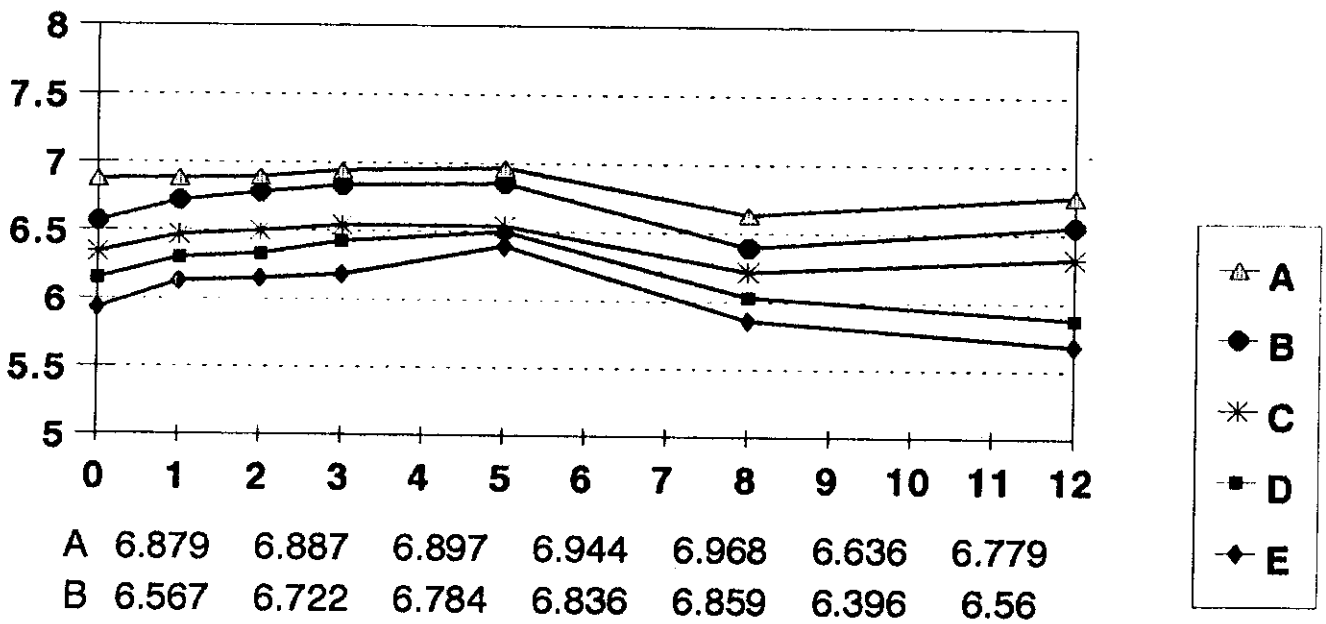


Figure (1). Changes in pH values of different lots during frozen storage at -20°C for 12 month. Lot A: 100 % minced hake; Lot B 75 % minced hake + 25 %minced sardine; Lot C: 50 % minced hake + 50 % minced sardine; Lot D: 25 % minced hake + 75 % minced sardine; Lot E : 100 % minced sardine.

pH



A	6.879	6.887	6.897	6.944	6.968	6.636	6.779
B	6.567	6.722	6.784	6.836	6.859	6.396	6.56
C	6.342	6.471	6.5	6.543	6.543	6.219	6.322
D	6.149	6.305	6.333	6.427	6.503	6.032	5.877
E	5.929	6.131	6.154	6.183	6.397	5.859	5.68

Months

variance of the values are presented in table (2), from which the inital pH value of minced hake (lot A) which is considered as one of low fat species, was significantly higher than the other lots. Same findings were observed at any given time of storage. During storage at -20°C no significant differences were found up to the end of storage period as seen in table (2). The higher pH value in the low fat species had been reported by other authors (Jimenez-Colmenero *et al* 1988; Huidobro and Tejada 1992). Ciarlo *et al* (1985) showed that the pH of frozen patagonian hake muscle rose was linearly with storage time.

The pH value of minced sardine (lot E) was the lowest one when compared with the other lots. However, up to the fifth month of storage at -20°C a slight increment of pH value was recorded, followed by a decrement at the end of storage period. Statistical analysis of the data given in table 2 indicated that significant differences were recorded from zero-time up to the first month of storage, after which no significant differences were found till the thrid month, while the significant differences were found up to the end of storage period. Samples of lot E was significantly different with other lots at any given time of storage. Suzuki and Watabe (1986) reported that control by of the muscle pH is important to prevent fish muscle proteins from denturation. They also mentioned that the pH of sardine usually ranged from 5.8 to 6.2. The lower pH detected in lot E could be related to the formation of lactic acid which was produced from glycogen by glycolysis in fish meat. On the other hand, the glycogen content muscle is highly concentrated in dark-fleshed fish and the lactic acid is also concentrated in the commercial fresh meat of these fish, exceeding 1000 mg/100 g in some species (Ikeda 1979).

With respect to lots B, C, and D, their pH values were intermediate between lot A and E depending on the ratios of

Table (2) Analysis of variance of pH value for the frozen minced samples during storage at -20°C for 12 months.

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	a/1	a/1	a/1	a/1	a/1	a/1	a/1
Lot B	a/2	b/2	c/12	d/2	d/2	e/2	a/2
Lot C	a/3	b/3	bc/3	c/3	c/3	d/3	a/3
Lot D	a/4	b/4	b/4	c/4	e/4	a/4	d/4
Lot E	a/5	b/5	b/5	b/5	c/5	a/5	d/5

* Different letters in each row indicate significant differences of storage period at ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage.

minced hake and minced sardine. For instance, in lot B which contain higher percentage of minced hake (75 %) and low percentage of minced sardine (25 %), the initial pH value was higher than in lots C and D. During storage at -20°C, a slight increment was noticed up to the fifth month, followed by a slight fall at the end of storage period. From the analysis of variance, given in table 2, a significant difference was found during frozen storage and lot B was significantly differed with other lots. Similar trends were noticed in lot C and D, while the values in lot D were lower when compared with lot C a trend which may be due to the presence of higher percentage of minced sardine (75 %) in the mixture.

The aforementioned results are in agreement with that of Rodger, *et al.*(1979) who showed that the pH of frozen cod fish fall down during the first week of storage, followed by a slight rise till week 12 and slight by refalling again.

An explantion for the rise in pH during frozen storage and its subsequent fall is perhaps easier to find if the reaction TMAO ----→ FA + DMA is considered. The production of DMA will lead to a pH rise since it is a basic compound. However, there will be a finite amount of TMAO which can be compose, and if DMA may diffuse from the tissue, at some point loss of DMA will exceed its production and hence the observed pH will fall. However, Poulter and Lawrie (1977) suggested that the increase in pH value of fish muscle during frozen storage could be probably due to the breakdown of urea to ammonia and accumulated with other basic products.

*** Dimethylamine:**

During frozen storage of gadoids such as cod and hake, trimethylamine oxide (TMAO) is enzymatically dissociated by

trimethylamine oxidase into dimethylamine (DMA) and formal_ dehyde (FA) at a rate dependent upon the applied temperature. Data given in figure 2 show the changes which took place in dimethylamine of different lots of minces fish as affected by frozen storage at -20°C for 12 months. Statistical analysis of the obtained results are given in tables (3, 4).

With respect to the mince hake (lot A) the available data proved that after freezing, the sample contained higher concentration of dimethylamine when compared with other lots, where the initial value was 0.598 μ mol/g mince and during storage at -20°C for 12 months its concentration tended to increase and reached to 10.94 μ mol/g minced hake. From the regression analysis in table (3) which preformed to obtain the best liner fit, a high correlation was determined (R= 0.9800) for lot A and significant differences at ($P<0.05$) were observed with all lots. Table 4 indicates the analysis of variance among values of DMA values according to storage period at -20°C for 12 months (letter in rows) and among different lots (numbers in each column) at any time of storage. It is clear from the data of lot A that ther was significant difference from zero-time until the end of storage period. At the begining of storage period there was a significant difference at ($P<0.05$) between lot A and different lots of minces; such trend was still going at any given time of storage up to the end of storage at -20°C. Similar results were found by Careche and Tejada (1991). Castell *et al* (1970) came to a similar conclusion, and showed that a significant increase in DMA concentration was observed in fillets of European (***Merluccius merluccius)*** stored at -18°C, and there was an increase in DMA in fillets stored at -24°C. They also reported that during deterioration of fish in the unfrozen state trimethylamine (TMA) accumulates rapidly, but very little amount of DMA was formed. Once the fish is frozen and goes into storage, DMA but not TMA

Table (3) Linear regression and analysis of variance of dimethylamine for lots stored at -20°C.

X-Y	R GENERAL/α	R_X/α	R_Y/α
A-B	0.9526/0.0000	0.9800/0.0000	0.9622/0.0000
A-C	0.9677/0.0000	0.9800/0.0000	0.9572/0.0000
A-D	0.9119/0.0000	0.9800/0.0000	0.9512/0.0000
A-E	0.5683/0.0001	0.9800/0.0000	0.9694/0.0000
B-C	0.9326/0.0000	0.9622/0.0000	0.9572/0.0000
B-D	0.8432/0.0000	0.9622/0.0000	0.9512/0.0000
B-E	0.5432/0.0000	0.9622/0.0000	0.9694/0.0000
C-D	0.9089/0.0000	0.9572/0.0000	0.9512/0.0000
C-E	0.5421/0.0000	0.9572/0.0000	0.9694/0.0000
D-E	0.5703/0.0001	0.9512/0.0000	0.9694/0.0000

Lot A: 100 % mince hake.

Lot B: 75 % mince hake + 25 % mince sardine.

Lot C: 50 % mince hake + 50 % mince sardine

Lot D: 25 % mince hake + 75 % mince sardine

Lot E: 100 % mince sardine.

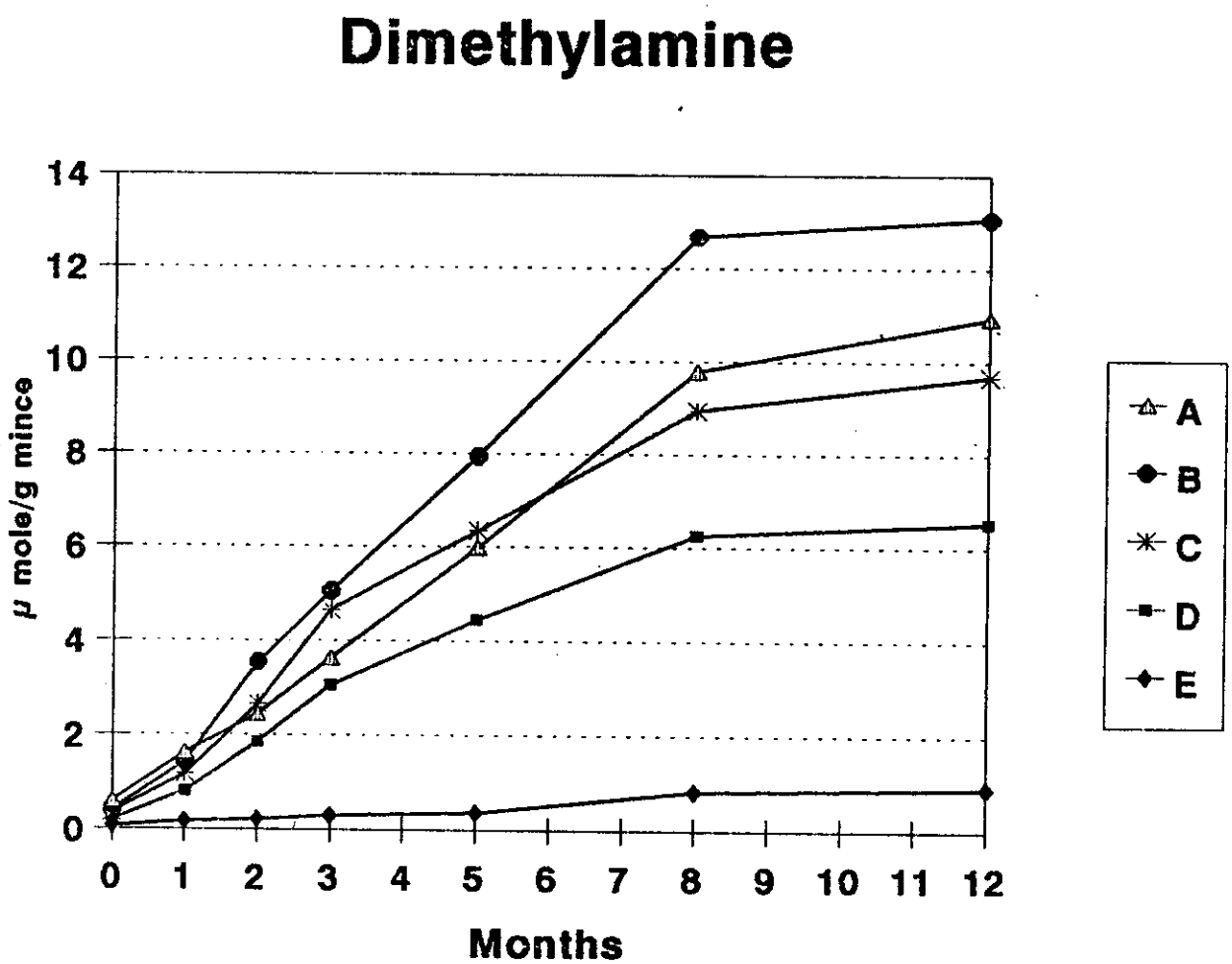
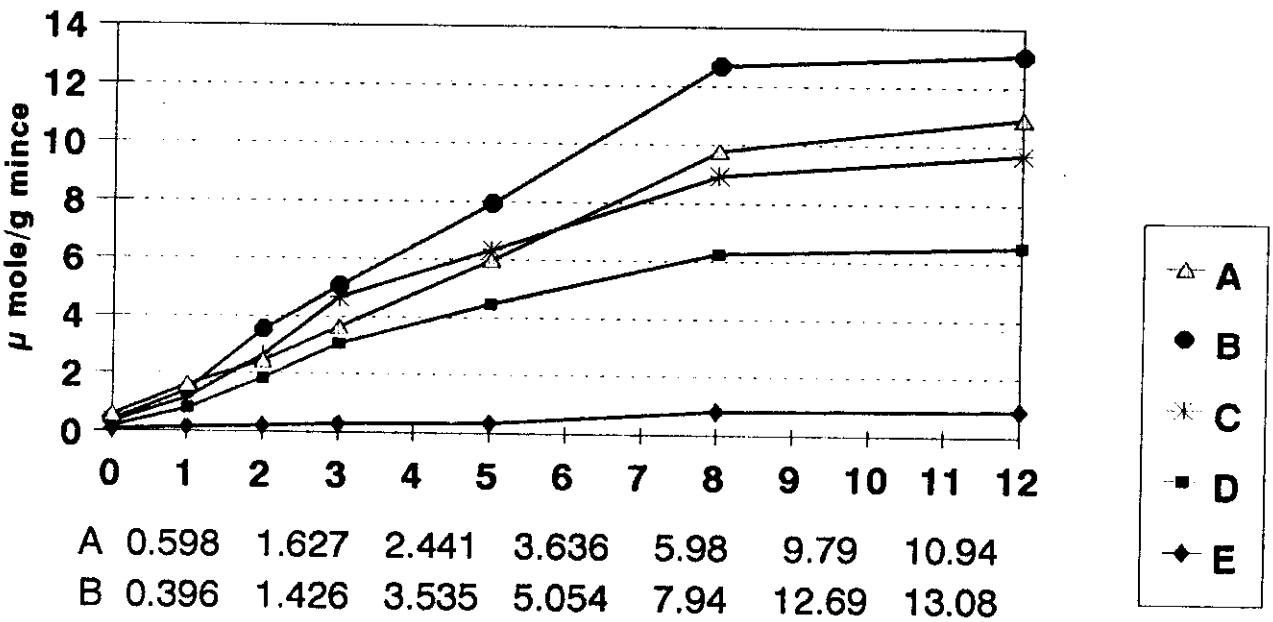


Figure (2). Changes in dimethylamine of different lots during frozen storage at -20°C for 12 months. Lot A: 100 % mince hake; Lot B: 75 % mince hake + 25 % mince sardine; Lot C: 50 % mince hake + 50 % mince sardine; lot D: 25 % mince hake + 75 % mince sardine; Lot E: 100 % mince sardine.

Dimethylamine



A	0.598	1.627	2.441	3.636	5.98	9.79	10.94
B	0.396	1.426	3.535	5.054	7.94	12.69	13.08
C	0.346	1.174	2.65	4.658	6.345	8.94	9.69
D	0.187	0.814	1.85	3.053	4.462	6.26	6.54
E	0.065	0.173	0.216	0.302	0.368	0.83	0.9

Months

Table (4) Analysis of variance of dimethylamine values for the frozen minced samples during storage at -20°C for 12 months.

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	a/1	b/1	c/1	d/1	e/1	f/1	g/1
Lot B	a/2	b/2	c/2	d/2	e/2	f/2	g/2
Lot C	a/3	b/3	c/3	d/3	e/3	f/3	g/3
Lot D	a/4	b/4	c/4	d/4	e/4	f/4	g/4
Lot E	a/5	b/5	b/5	c/5	c/5	d/5	e/5

* Different letters in each row indicate significant difference of storage period at ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage.

accumulates during deterioration. So, DMA content has been proposed as an index of frozen storage deterioration. Tokunaga (1970) found that, in Alask pollock fillet, the largest amount of DMA was produced at -10°C among the temperatures tested from -5 to -40°C. In the dark muscle, DMA reached 30 mg-N/ 100 g muscle after 10 days of storage at -10°C, while little or no TMA was detected. Such results are in parallel with the resules obtained by Castell *et al* (1973), i.e. no measurable amount of TMA was formed in frozen cod, pollock (*Pollachius uirens*), cusk (*Brosme brosme*) and silver hake (*Merluccius bilineris*) stored at -5°C, whereas DMA was formed in large quantities under the same conditions.

With respect to the changes that occur in dimethyl_ amine concentration of lot E (minced sardine) as a result of frozen storage at -20°C for 12 months, the data showed that at the begining of storage dimethylamine content was lower in minced sardine than all the other lots. By pro_ longing the storage period to 12 months at -20°C, the level of dimethylamine slightly increased from 0.065 to 0.90 μ mol/g minced sardine. Significant differences were recorded with other lots at any given time of storage (table 4). Careche and Tejada (1990 a) showed that the maximum DMA values for sardine detected after 370 days storage at -18°C was 0.86 mg N-DMA/100 g sample.

Changes in dimethylamine level in minced hake as affected by addition of different ratios of minced sardine during frozen storage at -20°C for 12 months were given in the same figure and tables.

As a result of adding minced sardine to hake (hake: sardine ratio 75:25 %) in lot B a significant reduction of the dimethylamine concentration was noticed at zero-time of storage when compared with lot A. The initial value for lot B was 0.396 μ mol/ g minced and after 12 months of storage

at -20°C the dimethylamine level of lot B showed ascending pattern of change and reached to 13.08 μ mol which is higher than lot A. Statistically significant differences at ($P<0.05$) were observed among lot B and other lots at any given time of storage at -20°C .

When 50 % of minced sardine was added to 50 % minced hake (lot C), the initial value of DMA level was 0.346 μ mol/g minced and on prolonging of frozen storage period, the dimethylamine concentration in lot C was progres_ sively increased to 9.69 μ mol/g minced fish at the end of storage period at -20°C. The previous results revealed that the concentration of dimethylamine was reduced when compared with the results of lot B as a results of adding 50 % 0f minced sardine in stead of 25 %. Table 3 showed the regression analysis from which ($R=0.9572$) and the significant differences at ($P<0.05$) were showed with other lots. There was a significant difference during frozen storage at -20°C (table 4), such trend was also noticed at any given time of storage between lot C and other lots.

In case of lot D; containing one part of minced hake (25 %) to three parts of minced sardine (75 %), its dimethylamine values followed the same trend, since the value changed from 0.187 to 6.54 μ mol/g minced by the end of storage. The lower dimethylamine values of lot D when compared with of lots B and C reflects the increment amount of minced sardine in the mixture minimized the formation of dimethylamine during frozen storage.

From the previous results, it could be observed that on prolonging frozen storage period of minced hake (lot A), the DMA concentration progressively increased. Such incre_ ment is attributed to decomposition of trimethylamine oxide (TMAO) to dimethylamine and formaldehyde by trimethylamine oxidase. These results are in agreement with those of Villarreal and Howgate (1991), they reported that many

species of gadoid fish are known to have an enzyme which splits trimethylamine oxide to DMA and FA in equimolecular amounts. They showed also that at -16°C DMA increased during frozen storage of cod fillets and after 766 days the DMA content was 11.8 mg DMA-N per 100 g fish. The deterioration of the quality of hake muscle during frozen storage has been associated with the formation of DMA and FA (Babbitt *et al* 1972). According to the opinion of Ciarlo *et al* (1985), increment of dimethylamine and formaldehyde would indicate the breakdown of trimethylamine oxide. This reaction could be the most important factor in muscle alteration leading to quality loss and determination of the shelf life of the product.

The lowest values of DMA obtained in minced sardine during storage, are agree with the fact that sardine fish is considered non DMA and FA forming species. When added minced sardine to minced hake (lot B) it could be noticed that at zero-time and up to the first month, dimethylamine concentration of lot B is lower than the corresponding values of lot A, while starting from the thrid month up to the end of storage period at -20°C, the hightest level of dimethylamine concentration was recorded for lot B. Such trend may be attributed to toughness of the tissue of lot A as shown in change of the texture (figure 4) and being more insoluble as shown in figure 8, and so more DMA formed not detected in lot A by the rough exprimental while more formaldehyde was observed (figure 3).

When the added amount of sardine to hake (lot C and D), a reduction in the concentration of DMA formation was noticed during frozen storage. Such trend is probably due to the fatty pattern of sardine fish, and to the fact that during frozen storage the lipids are more liable to oxidation. These oxidized lipids perhaps are acting as inhibitors of dimethylamine formation as suggested by Careche and Tejada (1990 a). They suggested, the existence

of an interference mechanism between lipid oxidation in fish muscle and the degrdation of trimethylamine oxide. In the trend, Sotelo, *et al* (1995) reported that addition of oxidized lipids to the minced fish causes inhibition of dimethylamine formation and also a loss of protein functionality during frozen storage. This would explain the decrease of the formation of dimethylamin during frozen storage when a high amount of sardine is added.

From the previous discussion, it could be concluded that on prolonging the frozen storage period of minced hake (lot A) the dimetyhlamine concentration progressively increased. The lowest values of dimethylamine were obtained in minced sardine during frozen storage. When more minced sardine is added to minced hake (lots C and D) a reduction in dimethylamine formation during frozen storage is observed.

* **FORMALDEHYDE (FA):**

Formaldehyde is one of the degradation products of trimethylamine oxide, formed during frozen storage of fillets or minced fish from gadoid fish species. Formaldehyde is very reactive to protein, can reduce considerably the level of solubility of the muscle proteins in sloutions containing NaCl or SDS (sodium dodecyl sulfate) (Rehbein and Horst 1985), and Del-Mazo *et.al.*(1994). For this reason, free FA detected in samples gives an idea of the amount that has not reacted in the muscle.

Figure (3) shows the changes in formaldehyde level of different lots for minced fish during frozen storage at -20°C for 12 months. Tables 5 and 6 showed the statistical analysis of the investigated different lots during frozen storage.

Regarding the linear regression and ANOVA (Table 5)

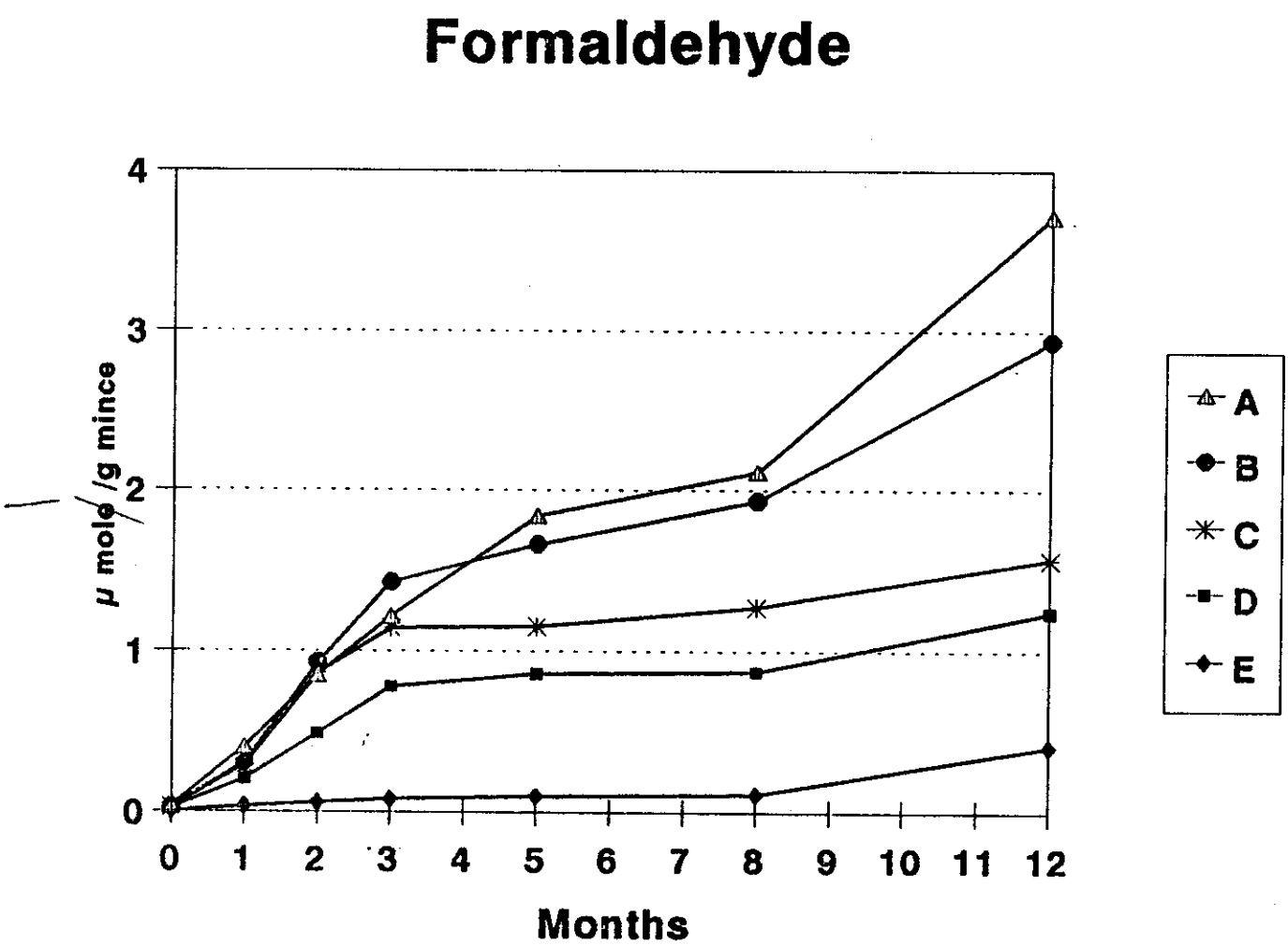
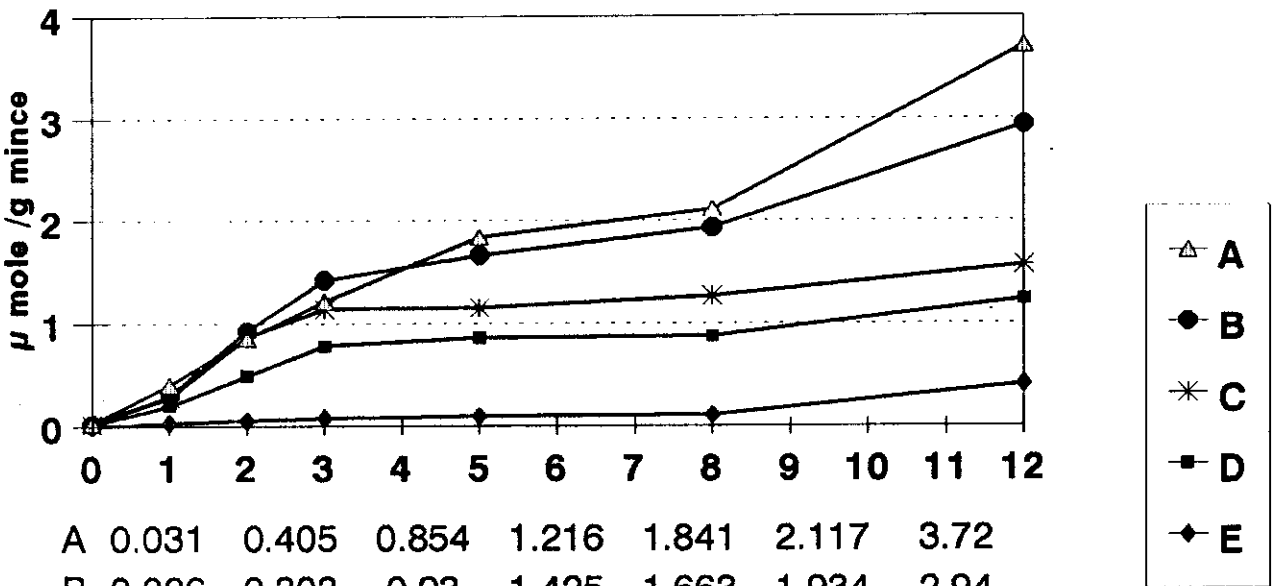


Figure (3). Changes in formaldehyde of different lots during frozen storage at –20°C for one year. Lot A: 100 % mince hake; Lot B: 75 % mince hake + 25 % mince sardine; Lot C: 50 % mince hake + 50 % mince sardine; Lot D: 25 % mince hake + 75 % mince sardine; Lot E: 100 % mince sardine.

Formaldehyde



A	0.031	0.405	0.854	1.216	1.841	2.117	3.72
B	0.026	0.303	0.93	1.425	1.663	1.934	2.94
C	0.024	0.284	0.865	1.143	1.154	1.271	1.57
D	0.019	0.203	0.49	0.783	0.862	0.876	1.24
E	0.001	0.034	0.056	0.08	0.098	0.109	0.41

Months

Table (5) Liner regression and analysis of variance of formaldehyde of lots stored at -20°C for 12 months.

X-Y	R GENERAL/α	R_I/α	R_I/α
A-B	o.9680/0.0000	0.9862/0.0000	0.9627/0.0000
A-C	0.8465/0.0000	0.9862/0.0000	0.8386/0.0000
A-D	0.7876/0.0000	0.9862/0.0000	0.9097/0.0000
A-E	0.5957/0.0000	0.9862/0.0000	0.9074/0.0000
B-C	0.8570/0.0000	0.9627/0.0000	0.8386/0.0000
B-D	0.7935/0.0000	0.9627/0.0000	0.9097/0.0000
B-E	0.5626/0.0001	0.9627/0.0000	0.9074/0.0000
C-D	0.8231/0.0000	0.8386/0.0000	0.9097/0.0000
C-E	0.5007/0.0007	0.8386/0.0000	0.9074/0.0000
D-E	0.6009/0.0000	0.9097/0.0000	0.9074/0.0000

Lot A: 100 % mince hake.

Lot B: 75 % mince hake + 25 % mince sardine.

Lot C: 50 % mince hake + 50 % mince sardine.

Lot D: 25 % mince hake + 75 % mince sardine.

Lot E: 100 % mince sardine.

the available data showed significant differences at ($P<0.05$) between different lots and the highest value of (R) was noticed in lot A (R = 0.9862) which, the lowest value was related to lot C (R = 0.8386).

At the begining of frozen storage, the same data proved that formation of formaldehyde was very low in all lots ranging from 0.031 in lot A to 0.001 μ mol/g minced in lot E. During storage at -20°C the incremental pattern for formaldehyde became more rapid and more pronounced in lot A which changed from 0.031 to 3.721 μ mol/g minced at the end of storage period. From table (6) statistical analysis proved that in lot A the significant differences were pronounced until the end of storage period. Kelleher, *et al* (1982) found that the level of free formaldehyde showed a general increase with storage time for frozen red hake blocks over a 6 months period at -18°C. Villarreal and Howgate (1991) found that a significant increase in FA concentration was observed in fillets of European hake (*Merluccius merluccius*) stored at -18°C.

In case of lot E (100 % minced sardine) the FA level increased slowly from an initial value of 0.001 to 0.421 μ mol/g minced fish. The significant difference was observed from zero-time up to the third month, but no significant differences was recorded between third and fifth month of storage at -20°C & after which the significant differences was re-recorded with increasing storage time.

With respect to lot B; addition of 25 % minced sardine to 75 % minced hake reduced formaldehyde concentration detected during frozen storage. In spite of Lot B showed a progressive increase in formaldehyde concentration as the time of storage prolonged from 0.026 at zero time to 2.940 μ mole/g minced at the end of storage, degree of increment is still less lower than lot A. The statistical analysis showed that significant differences were found between lot

B and other lots at any given time of storage. Also significant differences were shown during frozen storage.

Similar results were noticed in lot C when 50 % of minced sardine was added to 50 % of minced hake, but the increment in the ratio of the minced sardine to 50 % lead to higher decrease in formaldehyde formation during frozen storage when compared with lot A and lot B. The values increased progressively from 0.023 at the begining of storage reached to 1.572 μ mol/g minced fish after 12 months of storage at -20°C. Significant differences were observed up to the third month, and from the third month no-significant differences were observed until the eighth month, after which there is significant differences between eighth and twelfth month.

Regarding lot D; increase the ratio of minced sardine to 75 % of mixture cause a gradual increment in formaldehyde formation as affected by the prolonging of storage, where the initial value changed from 0.018 of to 0.876 μ mol after eighth month of storage at -20°C. However, the increment was more pronounced at the end of storage since the value reached 1.243 μ mol/g minced sample. From the pervious data, formation of formaldehyde was slightly occur in lot D during frozen storage at -20°C when compared with lots B and C.

Formation of formaldehyde during frozen storage dependens on trimethylamine oxide (TMAO) and TMAO demethy_ lase (TMAO-ase) enzyme which breakdown TMAO to formal_ dehyde and dimethylamine. So, in formaldehyde producing fish (lot A) the detrioration of fish flesh during frozen storage is faster compared with that of non-formaldehyde producing fish (lot E), where formaldehyde can hasten the denaturation of muscle protein during frozen storage. Such results were in agreement with Sotelo *et.al.*(1995) who

Table (6) Analysis of variance of formaldehyde for the frozen minced samples during storage at -20°C for 12 months.

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	a/1	b/1	c/1	d/1	e/1	f/1	g/1
Lot B	a/2	b/2	c/2	d/2	e/1	f/2	g/2
Lot C	a/2	b/3	c/3	d/3	d/2	d/3	e/3
Lot D	a/3	b/4	c/4	d/4	e/3	e/4	f/4
Lot E	a/4	b/5	c/5	d/5	d/4	e/5	f/5

* Different letters in each row indicate significant difference of storage period at ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage.

indicated that accumulation of FA takes place during frozen storage of some fish belonging to the order Gadiforms e.g. cod, pollack, haddock, whiting, hake and cusk. It has been shown that both FA and dimethylamine are products of the same enzyme-catalysed reaction, which has trimethylamine oxide (TMAO). TMAO demethylase (TMAO-ase), the enzyme which catalyses the breakown of TMAO to FA and DMA, has been located in the viscera and in the microsomal fraction of muscle, especially in dark muscle. Babbitt *et al* (1972) stated that the FA formed as a result of the reduction of TMAO to DMA during frozen storage of minced and intact hake (Merluccius products) muscle may be rapidly interacting with the myofibrillar protein which could explain, in part, the undesirable textural changes that occur during the frozen storage. However according to Ang and Hultin (1989) formaldehyde could increase the rate of protein denatu_ ration during frozen storage, which lead to aggregation of protein through noncovalent interaction. Rehbein (1981) reported that during frozen storage of fillets or minced flesh from fishes of the order Gadiformes, formaldehyde is produced by enzymatic decomposition of trimethylamine oxide. The concentration of the produced FA can rise to 70 m moles FA/kg wet weight depending on storage temperature and fish species. The FA concentration (being available to the muscle proteins) may be considerly lower than the measured amount of FA or dimethylamine, because fish muscle contains significant amounts of free amino acids, nucleo_ tides and creatine which are able to trap FA. Hultin (1992) mentioned that the amount of formaldehyde that is determined is always less than the amount perdicted from 1:1 ratio of DMA and formaldehyde expected. This phenomenon has generally been attributed to the loss of formaldehyde by reaction with a variety of chemical groups in muscle tissue. The formaldehyde is thought to react first with a group on one protein, such as the lysyl residue froming the hydroymethyl group which could interact with a group on another protein (such as another lysyl) to form the cross-

link.

The available data indicated the presence of a relation between addition of minced sardine to minced hake and reduction in formaldehyde formation during frozen storage at -20°C. The difference in the ratio of minced sardine (fatty species) which added to minced hake (lean species) was followed by the differences in formaldehyde producing during storage. From the previous data, the lower rate of formaldehyde formation in lot B, C, D may be attributed to minced sardine added which contain higher proportion of lipids lead to an inhibition of FA formation. Shenouda (1980) reported that, fish lipids are sussceptible to oxidize, particularly during frozen storage. The oxidation of lipids is probably due to the decrease in the formation of formaldehyde in fish muscle during storage. The obtained results are in agreemeant with that of Careche and Tejada (1990 a) who found lower rate of formaldehyde formation in lots of minced hake (*Merluccius merluccius* L.) mixed with added cod liver oil and oxidized cod liver oil when compared with control lot. They suggest that the protective role attributed to neutral lipids in semi-fatty and fatty species could be due to an inhibition of FA formation. Also they found lower formation of FA when the lots are more oxidized, a pattern which consider the role of oxygen in the inhibition of the TMAO degradation from a new perspective. Similar conclusion was given by Harada (1975) who showed that reducing conditions enhance and oxidizing conditions inhibit the enzymatic degradation of TMAO to DMA and FA. Sikorski and Kotakowska (1994) reported that lipids, especially oxidized lipids, may affect the hydrogen bonds and hydrophobic adherences in the proteins of frozen fish. The carbonyl groups of oxidized lipids may participate in covalent bonding, leading to the formation of stable protein-lipid aggregates. The role of lipid in freezing denaturation of proteins depends on the quantity and distribution of the lipids in the tissues, their

properties, and their hydrolysis and oxidation.

From the previous discussion, it could be concluded that on prolonging the frozen storage period of minced hake (lean species) the formaldehyde progressively increased. The results indicate that the samples with minced sardine showed reduction in formaldehyde formation during frozen storage at -20°C, while lot E had the lowest concentration in relation to other lots.

*** Texture**:

Evaluation of texture deterioration of fish during prolonged frozen storage can be done using the Instron Universal testing machine which can imitate the masticating action of humans to generate a texture profile analysis curve. On the other hand, texture of fish flesh is one of the most important factors that affecting the acceptability of fish products (Tsoladze, 1972).

Figure (4) shows shear resistance, measured with the Kramer shear cell, for the investigated lots of minces during storage at -20°C for 12 months. Statistical analysis of texture were also given in tables 7 and 8. Assuming linear regression and ANOVA of shear resistance (Table 7) showed that there were significant differences at ($p<0.05$) among different lots during storage at -20°C.

Regarding lot A (100 % minced hake) which forms formaldehyde and dimethylamine, the scored values of shear resistance that was 11.45 N at the begining of storage reaching 26.56 N after storage for 12 months at -20°C. From table 8 lot A showed a significant differences up to the second month, while no significant differences were observed between second and third month of storage at -20°C. It is also clear that there were significant differences between third and fifth month after which no

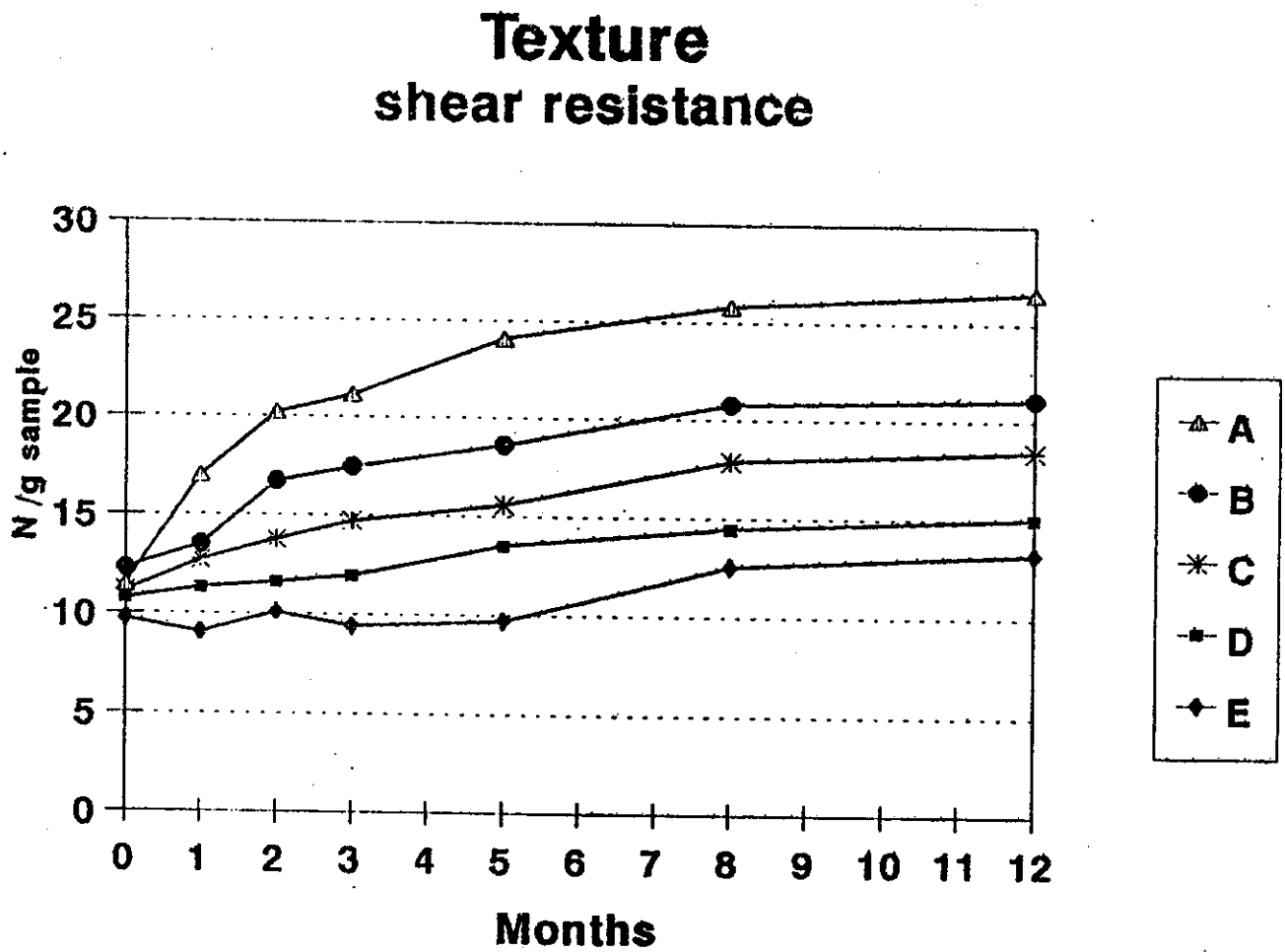
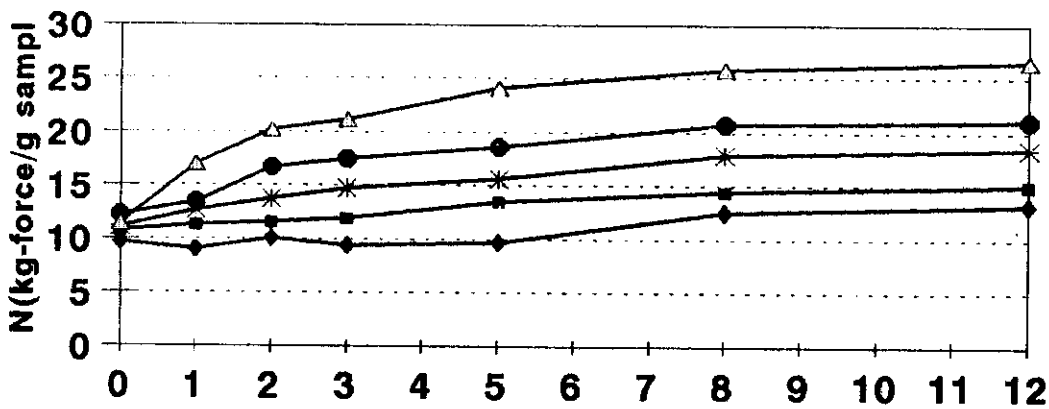


Figure (4). Changes in texture (shear resistance) in newtons (N) of different lots during frozen storage at -20°C for 12 month. Lot A: 100 % mince hake; Lot B: 75 % mince hake + 25 % mince sardine; Lot C: 50 % mince hake + 50 % mince sardine; Lot D: 25 % mince hake + 75 % mince sardine; Lot E: 100 % mince sardine.

Texture
shear resistance



A	11.45	17	20.23	21.17	24.07	25.82	26.56
B	12.26	13.48	16.71	17.47	18.62	20.78	21.12
C	11.12	12.69	13.76	14.72	15.6	17.89	18.46
D	10.76	11.31	11.61	11.95	13.5	14.46	15.05
E	9.7	9.06	10.09	9.41	9.71	12.54	13.23

Months

Table (7) Liner regression and analysis of variance of texture of lots stored at -20°C for 12 months.

X-Y	R GENERAL/a	R_I/a	R_I/a
A-B	0.7859/0.0000	0.8585/0.0000	0.9015/0.0000
A-C	0.6788/0.0000	0.8585/0.0000	0.9468/0.0000
A-D	0.5329/0.0003	0.8585/0.0000	0.9396/0.0000
A-E	0.4573/0.0026	0.8585/0.0000	0.8899/0.0000
B-C	0.8485/0.0000	0.9015/0.0000	0.9468/0.0000
B-D	0.6446/0.0000	0.9015/0.0000	0.9396/0.0000
B-E	0.5100/0.0000	0.9015/0.0000	0.8899/0.0000
C-D	0.8139/0.0000	0.9468/0.0000	0.9396/0.0000
C-E	0.6218/0.0000	0.9468/0.0000	0.8899/0.0000
D-E	0.7576/0.0000	0.9396/0.0000	0.8899/0.0000

Lot A: 100 % mince hake.

Lot B: 75 % mince hake + 25 % mince sardine.

Lot C: 50 % mince hake + 50 % mince sardine.

Lot D: 25 % mince hake + 75 % mince sardine.

Lot E: 100 % mince sardine.

Table (8) Analysis of variance of texture (shear resistance) for the frozen minced samples during storage at -20°C for 12 months.

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	a/12	b/1	c/1	c/1	d/1	d/1	d/1
Lot B	a/1	b/2	c/2	c/2	d/2	d/2	e/2
Lot C	a/13	b/2	cd/3	cd/3	d/3	e/3	e/3
Lot D	a/234	a/2	a/4	a/4	b/4	bc/4	c/4
Lot E	a/4	a/3	a/5	a/5	a/5	b/5	b/5

* Different letters in each row indicate significant difference of storage period at ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage.

significat differences until the end of storage period.

The lowest initial value was 9.7 N was that of lot E (100 % minced sardine) and no major changes were observed up to five months, after which shear resistance increased noticeably and reached 13.23 N. During frozen storage at -20°C no-significant differences were noticed in lot E up to fifth month of storage at -20°C followed by a significant differences between fifth and eighth month as seen in table 8. Similar values for hake and sardine were found by Careche and Tejada (1991).

Addition of minced sardine to minced hake reduced the changes which took place in shear resistance during frozen storage at different ratios. In lot B (75 % minced hake + 25 % minced sardine) a slight increment in shear resistance was observed at the commencement of storage when compared with lot A and then during storage of lot B a continuous increase in shear resistance was noticed but with lower rates in relation to lot A, where the values changed from 12.26 N to 21.12 N after 12 month of storage at -20°C.

The available results revealed also that in lot C which (consist of 50 % minced sardine + 50 % minced hake) a continuous ascending pattern in the resistance value took place. Such increment occurred but at lower values than lot A and lot B. However, the initial value of shear resistance for lot C at zero-time 11.12 N increased to 18.46 N at the end of storage period. In case of lot D (25 % minced hake in addition to 75 % minced sardine), where the values of shear resistance increased from 10.76 N at the begining of storage and reached 15.05 N at the end of storage period. The behaviour of shear resistance values showed no significant differences during frozen storage up to the thrid month, while significant differences were recorded between thrid and fifth month of storage at -20°C.

Analysis of variance between the different lots of minces fish at any given time of storage at -20°C are given in the same table. At the begining of storage there were no significant differences among lot A with lots B, C and D, while significant difference was found between B and D. From the second month there were significant differences among different lots up to the end of storage period. The variations among lots B, C and D may related to or responsed to the persentage of hake in the sample.

This textural changes which often decrease the water retention of muscle protein are probably due to the enzy_ matic breakdown of trimethylamine oxide to dimethylamine and formaldehyde (FA). The FA is then belived to react with the fish proteins to accelerate the undesirable texture changes, a pattern which indicate a reaction with low molecular weigh substances. Same findings were given by Samson and Regenstein (1986) who explaind that the more rapid textural deterioration can be attributed to enzymatic degradation of trimylamine oxide (TMAO) into formaldehyde and dimethylamine during frozen storage in cod. The FA react with proteins and formed crosslinks at certain sites within fish muscle structure and may contribute to the textural toughness.

From the obtained results it could be observed that, in spite of the shear resistance of lot A increased faster than that of the other lots, lot A had more FA and less DMA (figure 2). The less detected amount of DMA may be attributed to the toughness of the tissue and more insoluble and so not detected by the rough exprimental. such conclusion is giving with Haard (1992 a) who reported that for gadoids, there exists a positive relationship between texture toughening and DMA formation during frozen storage. However, textural toughening of fish during frozen

storage can result from a variety of mechanisms including ice crystal damage, lipid-protein interaction, salting out of proteins, free fatty acid-protein interaction, and formaldehyde-protein interactions (Lundstrom *et al*.1981). In certain gadoid species, including hake, the latter mechanism is assumed to play the major role in textural toughening. A comparison between filltes of different gadoid species has shown that at level representative of the normal range, formaldehyde insolubilize more protein than did free fatty acids (Careche and Tejada 1990 a). Jarenbäck and Liljemark (1975) stated that the development of tough and dry texture during frozen storage of certain species of fish has been attributed to changes within their myofibrillar proteins. The texture characteristics are shown to change rapidly at -10°C, moderatly at -20°C and not appreciably at -30°C. Finding were found by Crawford *et al* (1979) who noticed a significant correlation between sensory evaluation and the DMA and FA levels of minced pacific hake (Merluccius products) frozen at -20°C.

Generally from the above mentioned results it could be concluded that there was an increase in the texture of investigated minced fish samples during storage at -20°c. Furthermore, in a formaldehyde forming species (lot A) the obtained values were higher than in non-formaldehyde species (lot E). On contrary, addition of minced sardine to minced hake lead to the reduction in the value of shear resistance and the highest reduction in toughening was observed in lot D. So decrease in the shear resistance due to the increase of the added percentage of minced sardine in the mixtures, is probably due to the presence of sardine lipids during storage, beacuse lipid inhibit formation of dimethylamine and formaldehyde as reported by Careche and Tejada (1991). They studied the effect of added cod liver and oxidized cod liver on the measurement of texture in minced hake (***Merluccius merluccius*** L.), megrim

(*Lepidorhombus whiffiagonis* W.) and sardine (*Sardine pilchardus* W.) during frozen storage at -18°C. The results showed that addition of neutral and oxidized lipids even at high rancidity level, do not affect shear resistances which measured by the Kramer shear-compression cell in non-formaldehyde forming species such as megrim and sardine, over the whole frozen storage period. However, in a formal_ dehyde forming species such as hake, in the presence of neutral and oxidized lipids at the end of the storage period, the value of shear resistance was lower than in the absence of these lipids, probably owing to formation of less formaldehyde in the latter cases. Another possible explanation is that in this case the decrease of shear resistance when the percentage of minced sardine increase in the mixture cannot be attributed to a lower amount of FA formed, but to a different mechanisms when sardine muscle is present. Sotelo *et al.* (1995) found the same trend where addition of oxidized lipids to the mince causes inhibition of DMA formation and also a loss of protein functionality during frozen storage. Rehbein and Orlick (1990) also concluded that the concentration of malonadehyde (MA) and other products of oxidative lipid degradation (peroxides, aldehydes, ketones, etc.) is too low to denature signi_ ficant amount of fish muscle proteins during frozen storage of the investigated lean species.

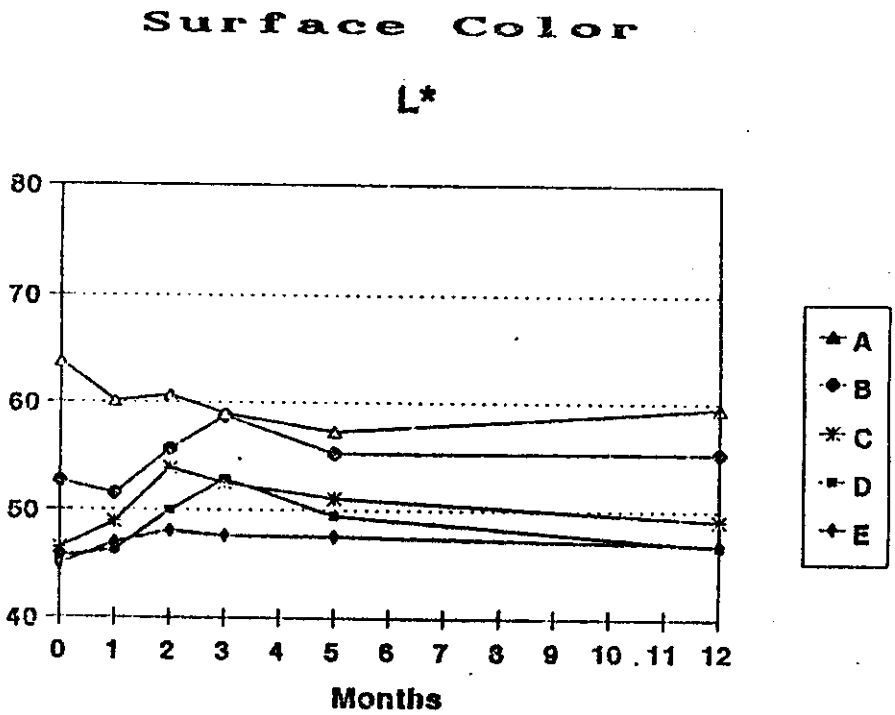
* **color** :

To define the color of an object quantitatively, three fundamental parameters must be specified. They are: hue or spectral color, which identifies the object as red, green, yellow, blue, or an intermediate color between these; Saturation or purity, which is the strength or intensity of the hue; and lightness or luminance, which is the amount of light reflected or transmitted from the object. In this work data expressed these measurements as L*, a*, and b* color values. The L* value measures lightness, or the

amount of light reflected or transmitted by the object. The a* and b* values are the chromaticity corrdinates from which information about hue and saturation can be obtained. The a* value measures redness when plus and greenness when minus. The b* value measures yellowness when plus and blueness when minus.

Data given in figures (5, 6, 7) show the changes which took place in the surface and in the internal color during frozen storage of different lots of fish minces. Tables (9, 10, 11) shows the analysis of variance of the same samples under the same storage condition. The lightness (L*) values of lot A (100 % minced hake) was higher when comparing with other lots, where significant differences were observed among lot A and the other lots. Such trend was shown at any given time of storage in either surface or the internal color. During frozen storage, the lightness on the surface decreased slowly and similar pattern was noticed in the internal lightness. From statistical analysis significant differences were observed for L* value on the surface of lot A between zero-time and the first month, after which, no significant differences were recorded up to the end of storage period. These finding were noticed for the internal lightness. On the other hand, lot A was more greenness (a*) (figure 6) and less yellowness (b*) (figure 7) when compared with other lots, where the significant differences were recorded among lot A and other lots within the tested position.

Regarding the measurements of the surface and the internal color of lot E (minced sardine), the lightness values (L*) (Fig. 5) indicated that lot E had less lightness and a notable slight increase occurred on the surface after one month of frozen storage. No significant differences were found from the second month till the end of storage at -20°C, where the values of L* was nearly constant. The L* value in the internal sample displaying



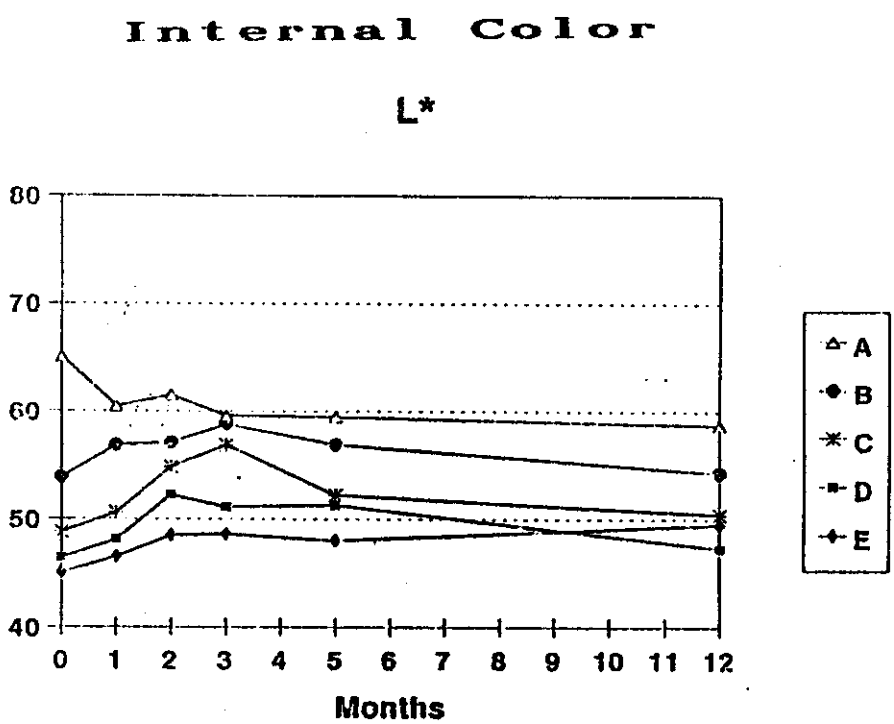
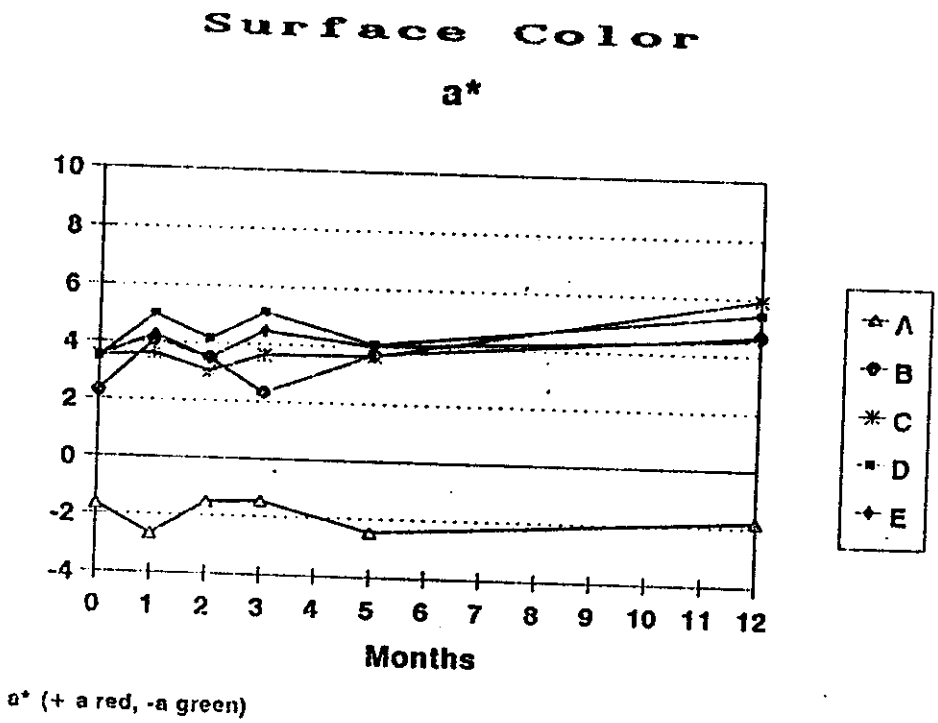


Figure (5). Changes in lightness (L*) in either surface and internal samples stored at -20°C for 12 months. Lot A: 100 % minced hake: Lot B: 75 % minced hake + 25 % minced sardine ; Lot C: 50 % minced hake + 50 % minced sardine; Lot D: 25 % minced hake + 75 % minced sardine; Lot E: 100 % minced sardine.



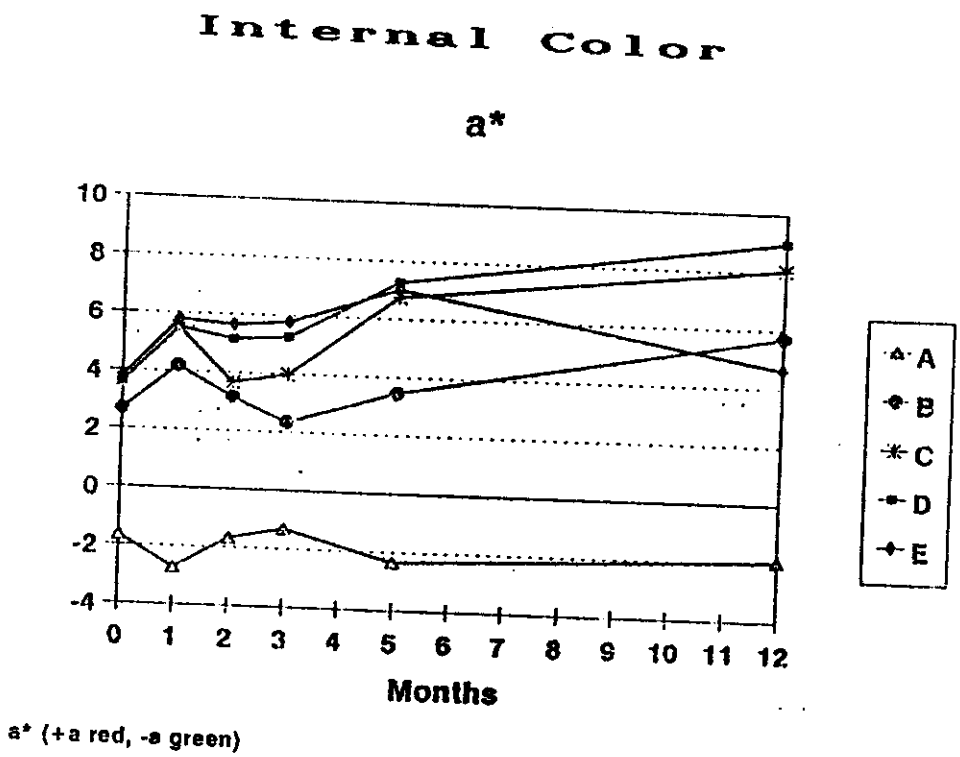
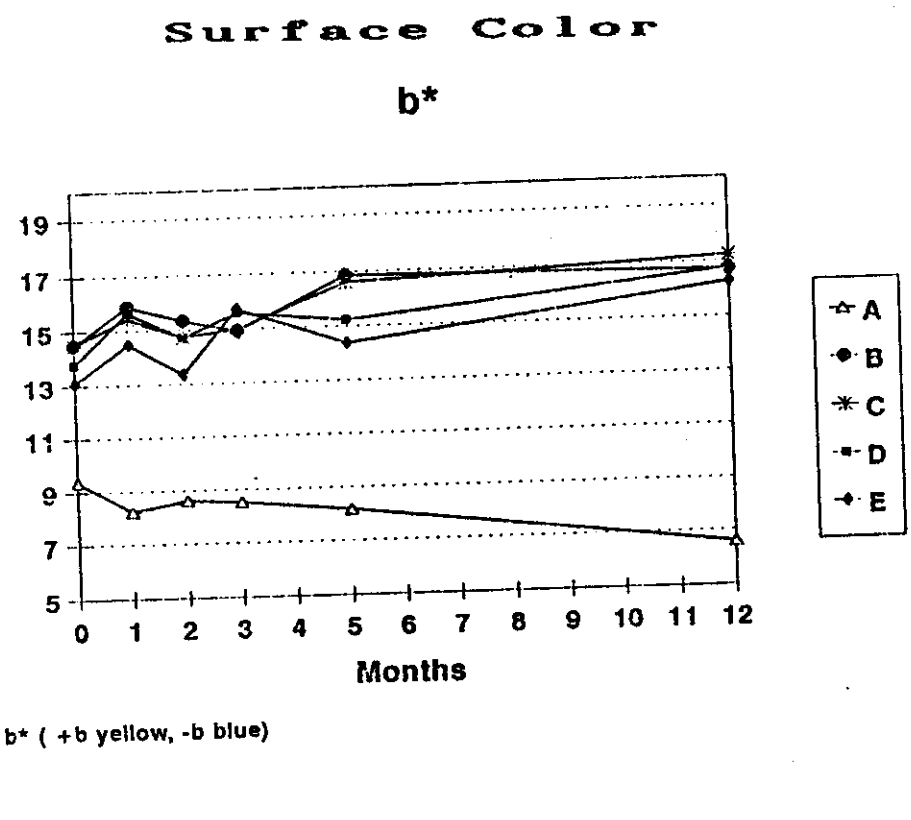


Figure (6). Changes in redness (a*) in either surface and internal samples stored at -20°C for 12 months. Lot A: 100 % minced hake: Lot B: 75 % minced hake + 25 % minced sardine ; Lot C: 50 % minced hake + 50 % minced sardine; Lot D: 25 % minced hake + 75 % minced sardine; Lot E: 100 % minced sardine.



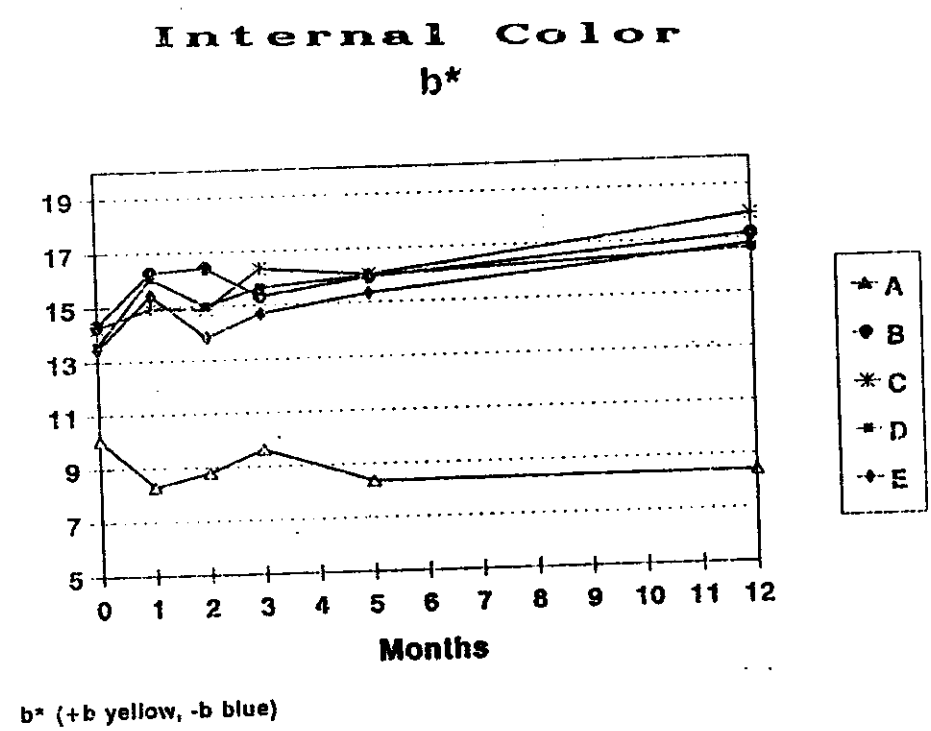


Figure (7). Changes in yellowness (B*) in either surface and internal samples stored at -20°C for 12 months. Lot A: 100 % minced hake: Lot B: 75 % minced hake + 25 % minced sardine ; Lot C: 50 % minced hake + 50 % minced sardine; Lot D: 25 % minced hake + 75 % minced sardine; Lot E: 100 % minced sardine.

Table (9) Analysis of variance of redness (a*) in either surface and internal samples stored at -20°C for 12 months.

Surface redness (a*)

Month	0	1	2	3	5	8	12
Lot A	a/1	b/1	a/1	a/1	b/1	c/1	a/1
Lot B	a/2	bd/23	c/2	a/2	bc/2	a/2	d/2
Lot C	a/3	a/2	a/2	a/3	a/2	a/3	b/3
Lot D	a/3	bc/3	ab/3	bc/4	ab/2	ab/3	c/23
Lot E	a/3	bc/23	a/2	bc/5	abc/2	ab/3	c/2

Internal redness (a*)

Month	0	1	2	3	5	8	12
Lot A	a/1	b/1	ac/1	d/1	e/1	d/1	c/1
Lot B	ab/2	cd/2	abe/2	a/2	bd/2	de/2	f/2
Lot C	a/2	b/3	a/3	a/3	c/3	ab/3	d/3
Lot D	a/2	b/3	b/4	b/4	c/3	c/4	d/4
Lot E	a/2	b/3	b/5	b/5	c/3	b/5	ab/5

* Different letters in each row indicate significant differenceas of storage period ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage

Table (10) Analysis of variance of yellownesss (B*) in either surface and internal samples stored at -20°C for 12 months.

Surface yellowness (b*)

Month	0	1	2	3	5	8	12
Lot A	ab/1	c/1	ac/1	ac/1	c/1	b/1	d/1
Lot B	a/2	b/2	b/2	a/2	c/2	a/2	c/2
Lot C	a/2	ab/23	a/2	a/2	bc/2	d/3	c/2
Lot D	ab/2	ac/23	a/2	ac/2	ac/3	b/3	c/2
Lot E	ab/2	ac/3	abd/3	cd/2	ac/3	b/1	c/2

Internal yellowness (b*)

Month	0	1	2	3	5	8	12
Lot A	ab/1	c/1	ac/1	abc/1	c/1	b/1	c/1
Lot B	a/12	bd/2	b/2	ab/23	bd/2	bd/2	d/2
Lot C	a/12	a/3	a/3	ab/4	ab/2	a/3	b/2
Lot D	a/12	ab/24	ab/3	ab/24	ab/2	ab/13	b/2
Lot E	abc/2	ac/35	ab/4	abc/3	ac/2	b/4	c/2

* Different letters in each row indicate significant differenceas of storage period ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage

Table (11) Analysis of variance of lightness (L*) in either surface and internal frozen minced samples stored at -20°C for 12 months.

Surface lightness (L*)

Months	0	1	2	3	5	8	12
Lot A	ab/1	cd/1	ad/1	d/1	d/1	b/1	d/1
Lot B	ab/1	b/2	ac/2	cde/1	a/2	d/2	ae/2
Lot C	a/2	ab/3	bc/2	bc/2	ac/3	ac/3	ac/3
Lot D	a/2	a/4	c/3	d/2	c/4	c/4	a/3
Lot E	a/2	a/5	a/4	a/3	a/5	a/4	a/3

Internal lightness (L*)

Months	0	1	2	3	5	8	12
Lot A	ab/1	c/1	bc/1	c/1	c/1	ad/1	c/1
Lot B	a/2	ab/2	ac/2	bcd/2	ade/2	bce/2	a/12
Lot C	a/23	ab/3	cd/23	d/3	bce/3	bce/3	ae/23
Lot D	a/3	a/4	b/3	b/4	b/3	ab/4	a/3
lot E	a/3	ab/4	ab/4	ab/5	ab/4	ab/5	b/23

* Different letters in each row indicate significant differences of storage period at ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage.

the same results. From a* values data showed that minced sardine was more red in color when compared with lot A. The yellowness (b*) in both the surface and the internal sample were not significantly different and the investigated sample was more yellow at the end of storage period.

In case of lot B, and as a result of adding 25 % of minced sardine to 75 % minced hake lightness degree (L*) decreased when compared with lot A. During frozen storage a slight increase occurred in the lightness of the surface and there were no differences between the surface and the internal lightness. The sample turned redder and more yellow when compared with lot A with a significant higher a* and b* values at the end of storage. No noticeable differences were observed between both the surface and the internal color of lot B.

With respect to lot C, data showed a decrement trend in lightness (L*) which still going on by prolonging the frozen storage period. The degree of significant differe_ nces were recorded among lot C and (Lots A and B) within most of the storage period. The lightness (L*) of lot D was lower in relation to other mixed lots and the L* value increased slightly during frozen storage. The degree of red color (indicated by a* value) means that the sample had red color and frozen storage tend to increase in the a* value, however, the surface showed less red color than the internal of lot D. The yellowness (b*) did not differ among mixture and the result showed that no differences between the b* value of the surface and the internal sample.

From the available data in table (12) it could be observed that both surface and internal color of lot A (100 % minced hake) exhibiting higher color differences (ΔE) and lower chromaticity differences (ΔC) at the end of storage period.

Table (12) Differences of total color (ΔE) and chroma (ΔC) of lots between zero-time and samples stored at -20°C for 12 months.

Lot	Total color difference (ΔE)		Chromaticity differences (ΔC)	
	Surface	Internal	Surface	Internal
A	5.14	6.56	2.78	1.66
B	4.13	4.20	3.13	4.17
C	4.49	5.96	3.64	5.74
D	3.75	6.24	3.59	6.18
E	3.90	5.66	3.37	3.45

$$\Delta E = \sqrt{(L_1^*-L_0^*)^2 + (a_1^*-a_0^*)^2 + (b_1^* -b_0^*)^2}$$

$$\Delta C = \sqrt{(a_1^* - a_0^*)^2 + (b_1^* - b_0^*)^2}$$

Where:- L_1^*, a_1^*, b_1^* the coordinates CIELAB of samples stored for 12 months at -20°C and L_0^*, a_0^*, b_0^* the coordinates at zero-time of storage.

Generally from the obtained result it could be observed that there were significant changes in the L*, a* and b* values between lot A (100 % minced hake) and lot E (100 % minced sardine) where sardine minces had less lightness, more redness and yellowness, while hake mince was lighter, less red and yellow. Variations in the L*, a* and b* between both lots A and E was due to minced sardine which had a large amount of dark muscle, since the dark muscle is red-colored due to high pigments content such as homoglobin and myoglobin. Suzuki (1981) mentioned that small pelagic fishes, such as sardine and mackerel, were generally rich in dark muscle and the red color of hemoproteins, hemoglobin (Hb) and myoglobin (Mb). The concentration of Mb is up to 300-600 mg/100 g of the fish dark muscle, where mackerel and sardine contain 600 and 375 mg/100 g of the dark muscle respectively. From the prepared mixtures, it could be concluded that addition of minced sardine (dark muscle) to minced hake lead to a noticeable reduction in the lightness(L*), and samples turned redder (a* increased) and more yellow (b* increased). These changes are more evident in lots C and D.

During frozen storage and thawing, the changes in the L*, a*, and b* values occur, where Fevnandez and Berry (1989) stated that the color changes may be caused by the oxidation state of heam pigments, or by the interaction of amines and sugars in "Maillard reaction", which are a major cause of browning in a prolonged storage period. Shenouda (1980) reported that undesirable changes in color during frozen storage is due to the changes that occure in muscle proteins or protein-bound pigment, or to changes in certain pigmented protein. For example, conversion of metaglobin and oxymyoglobin to metamyglobin, usually occur in frozen tuna meat. Yellowing which occurs as a result of prolonging storage of frozen fish could associated with lipid oxidation and carbonylamine reaction as mentioned by Haard (1992 b) and Boon (1977).

Part II: Effect of frozen storage at -20°C on the functional properties of proteins of minced fish:

One of the consequences of protein denaturation is the change in their functional properties which are usually primary factors determing their utility in food products. Some of the most important functional properties in fish muscle are protein solubility, viscosity, emulsifying capacity and emulsion stability.

* **Changes in protein solubility** :

Figure 8 shows the influence of frozen storage on the protein solubility of the different mince lots. Table 14 gives the analysis of variance among mean values of protein solubility according to storage period and at any given time of storage.

Regression analysis and analysis of variance at ($p<0.05$) show that lots E and D had the highest value of the correlation coefficient (R) (0.8409 and 0.8017, respectively) followed by lots A, C and B where the (R) value of the tested frozen samples seemed to be similar in these lots as seen in table 13. The data also proved the presence of significant differences within the different lots during frozen storage.

The percentage of soluble protein did not initially show any significant differences among lots A, B, and C (Table 14), while the highest initial soluble protein level was noticed in lot E, being lot D intermediate among the previous lots. During storage at -20°C the soluble protein level of the control A decreased sharply up to the third month and seemed to be stable until the end of storage period. The decline in the protein solubility was found to be quite rapid and was more pronounced for lot A when compared with the other lots. Similar pattern was observed

Table (13) Liner regression and analysis of variance of protein solubility of lots stored at -20°C for 12 months.

X-Y	R GENERAL/α	R_X/α	R_Y/α
A-B	0.6410/0.0000	0.6843/0.0009	0.6245/0.0025
A-C	0.6426/0.0000	0.6843/0.0009	0.6755/0.0008
A-D	0.6572/0.0000	0.6843/0.0009	0.8017/0.0000
A-E	0.5292/0.0004	0.6843/0.0009	0.8409/0.0000
B-C	0.6432/0.0000	0.6245/0.0025	0.6755/0.0008
B-D	0.6753/0.0000	0.6245/0.0025	0.8017/0.0000
B-E	0.5389/0.0002	0.6245/0.0025	0.8409/0.0009
C-D	0.7245/0.0000	0.6755/0.0008	0.8017/0.0000
C-E	0.6020/0.0000	0.6755/0.0008	0.8409/0.0000
D-E	0.7299/0.0000	0.8017/0.0000	0.8409/0.0000

Lot A: 100 % mince hake.

Lot B: 75 % mince hake + 25 % mince sardine.

Lot C: 50 % mince hake + 50 % mince sardine.

Lot D: 25 % mince hake + 75 % mince sardine.

Lot E: 100 % mince sardine.

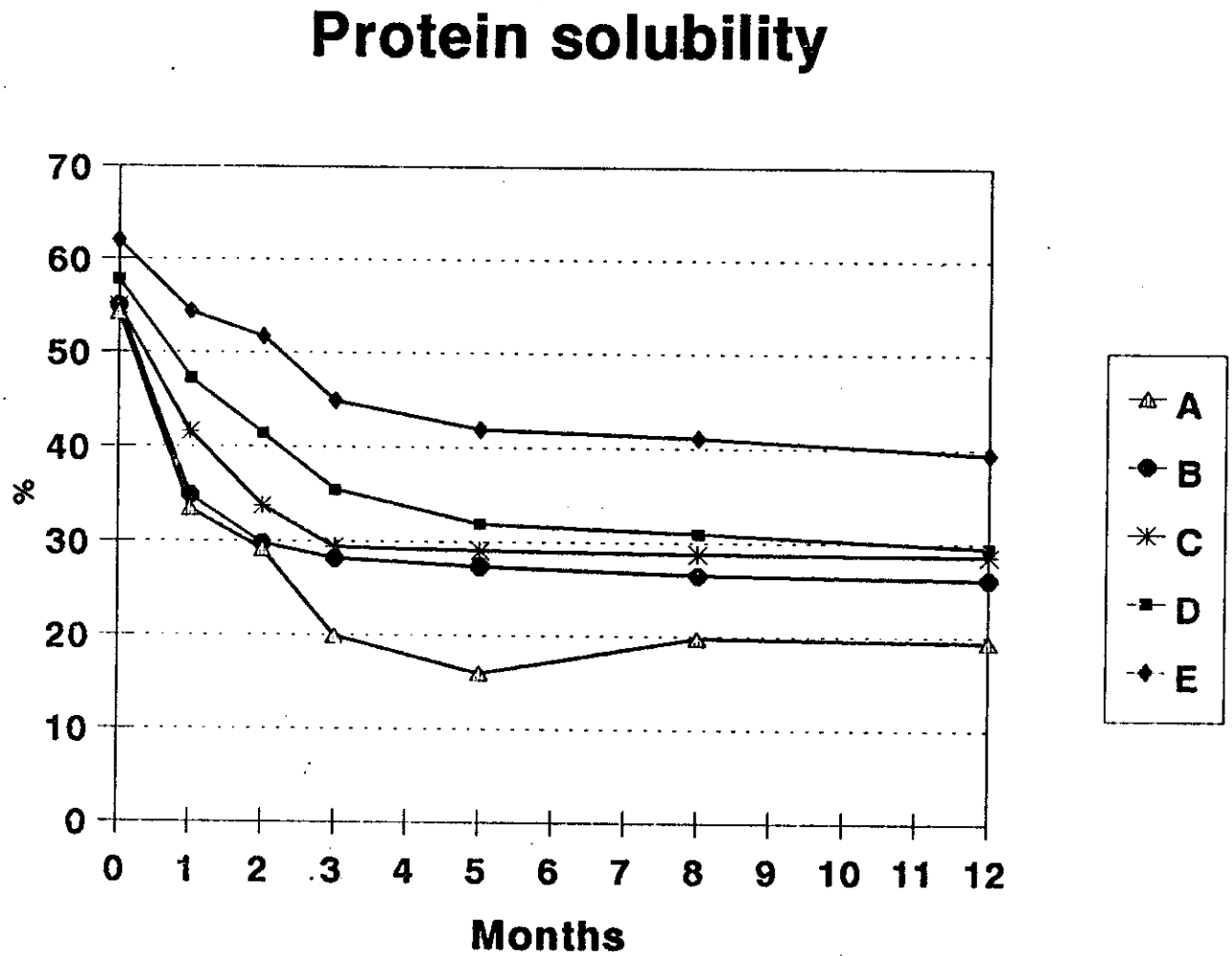
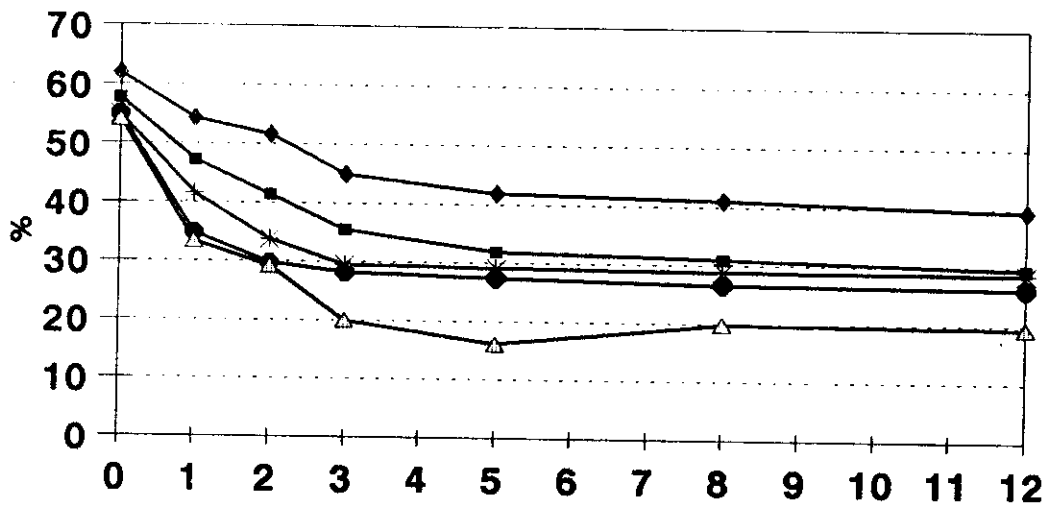


Figure (8). Changes in protein solubility of different lots during frozen storage at –20°C for one year. Lot A: 100 % mince hake; Lot B: 75 % mince hake + 25 % mince sardine; Lot C: 50 % mince hake + 50 % mince sardine; Lot D 25 % mince hake + 75 % mince sardine; Lot E: 100 % mince sardine.

Protein solubility



A	54.17	33.41	29.17	19.96	16.04	19.8	19.43
B	54.95	34.86	29.76	28.21	27.38	26.55	26.17
C	54.93	41.6	33.75	29.42	29.07	28.79	28.64
D	57.83	47.28	41.45	35.51	31.91	30.95	29.51
E	61.95	54.38	51.65	44.94	41.9	41.08	39.47

Months

Table (14) Analysis of variance of protein solubility for the frozen minced samples during storage at -20°C for 12 months.

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	a/1	b/1	c/1	d/1	e/1	d/1	d/1
Lot B	a/1	b/1	c/1	c/2	c/2	c/2	c/2
Lot C	a/1	b/2	c/32	d/2	d/23	d/23	d/2
Lot D	a/12	b/3	bc/3	cd/3	d/3	d/3	d/2
Lot E	a/2	b/4	b/54	c/4	cd/4	d/4	d/3

* Different letters in each row indicate significant difference of storage period at ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage.

in hake mince (De Koning *et al.* 1985). Tejada *et al.* (1984) stated that the decline in protein solubility was found to be rapid in cod at the storage temperature employed -12°C, practically reaching the minimum level after 60 days. Same finding were also mentioned by Abdullah and Yu (1985) and King and Poulter (1985).

The changes of protein solubility of lot E are given in the same figure. A reduction in protein solubility was also observed during storage until the end of storage period. In spite of the decrement pattern of the protein solubility of the minced sardine, it maintained as the largest soluble protein when compared with minced hake. From table (14) the statistical analysis showed that there were significant differences between zero-time and up to the third month, while no significant differences were found from the fifth month until the end of storage.

In lot B when added 25 % of minced sardine to 75 % minced hake, the soluble protein was similar with lot A up to the second month, afterwards the solubility was higher in the mixed lot than lot A. Significant differences ($p<0.05$) were found from zero-time until the second month, after which no significant differences were noticed until the end of storage.

Similar trend was found in lot C, but when the amount of minced sardine added to minced hake increase to 50 % a gradual decrease in solubility is observed with maintained the largest amounts of soluble protein when compared with lot A and B. Statisticaly, the values were significantly different till the second month and in the remainder of the storage period; no significant differences were noticed.

Regarding lot D which contained a higher amount of minced sardine (75 %), the initial soluble protein level was higher than the other mixtures. A lower decremental

trend of solube protein was noticed during frozen storage in relation to the corresponding values of lot B and C. From statistical view points significant differences were found within the storage period that extended to the third month, while no significant were observed till the end of storage period.

The downward trend in protein solubility in lot A could be attributed to the formation of formaldehyde from trimethylamine oxide products by enzymatic demethylation. The formaldehyde was formed during storage and react with proteins and accreleate protein aggregation (Del mazo *et al*, 1994 and Gill *et al*.1979). Other possible explanation of the downward trend of protein solubility was based on the opinion of Srikar and Reddy (1991). They stated that the decrease in the solubility of the proteins of the frozen stored pink perch (*Nemipterus Japonicus*) minced (lean variety of fish) was possibly attributable, or partially so, to the low neutral lipids (e.g. triglyceride) content. Neutral lipids have the capacity to dissolve free fatty acids (FFA) and neutralise their hydrophobic; i.e. desolubilising effect on the protein.

The differences in protein solubility between minced hake and minced sardine were due to the fact that sardine is one of species that are non-producing formaldehyde during frozen storage. Huidobro and Tejada (1992) showed that there was a general downward trend in solubility which was more pronounced in blue whiting (BW) than in horse mackerel (HM) or mackerel (M). Such trend may be attributed to protein aggregation caused by the formaldehyde generated in BW muscle during frozen storage, since it was the only one of the three species attain high dimethylamine and formal_ dehyde concentration. Careche and Tejada (1990 b) postu_ lated that the stability of semi-fatty species, which do not form FA and DMA in large quantities during frozen storage, is due to the action of neutral lipids

which, at proper concentration and location, can produce a protective effect on fish proteins. In the same sequence Jiménez-Colmenero and Borderias (1983) stated that during frozen storage at -20°C for 8 months, decrease in protein solubility was more pronounced for blue whiting than horse mackerel.

The general reduction trend in the decrease of protein solubility as a result of addition of minced sardine indicate a reversible relation between the formaldehyde produced during frozen storage and the addition ratios of minces which contain neutral lipids. This protective role could be due to an inhibition of formaldehyde formation. The aformentiond results are in agreement with Stodolnik and Knasiak (1984) who proved that lipid contents in fish flesh have influenced significantly on the solubility of proteins during freezing. Fish flesh containing 4 and 8 percent of lipids characterized by the least level of myofibrillar proteins denaturation. Same findings were mentioned by Careche and Tejada (1990 a) who studied the effect of added neutral and oxidized lipids on protein solubility of minced hake. They found a gradual decrease in solubility during frozen storage in control sample. Samples with neutral and oxidized lipids added do not show signi_ ficant differences between them during frozen storage. Protein solubility of these samples was different from that of the control, being lower in the first month and higher in the remainder of the storage period.

From the previous discussion it could be concluded that minced sardine characterized by high level of soluble protein. On prolonging of the frozen storage period the downward trend in protein solubility was found in all samples, while the declin that was more pronounced in lot A was mainly extended until the fifth month. With respect to the addition of minced sardine containg neutral lipids to minced hake; it caused more stability with lower of

denaturation and protein aggregation and so, contained the large amounts of soluble protein. The highest reduction in protein solubility observed in lot B as well as the noticeable variations in soluble protein of lots B, C and D could be related to the different ratios of minced sardine which added to minced hake. However from 5th month of storage no significant changes were observed among the three mixed lots.

*** The total protein content of the mince homogenate:**

Figure (9) shows the influence of frozen storage at -20°C for 12 months on the total protein in the homogenate of different lots after filtering through cheese cloth.

Data of table (15) show the R value (regression analysis) and analysis of variance at ($p<0.05$). The higher value of R was more pronounced in lot A (R=0.9515), while the lower value was recorded for lot E (R=0.6028). The R values of lots B, C, and D semed to be similar being (0.8411, 0.8959, 0.8808, respectively). From the same table the ANOVA analysis showed that there were significant differences among lots.

From figure 9 and table 16 data indicated that after freezing, the homogenate of minced hake (lot A) contains lower level of total protein when compared with other lots. The initial value of total protein was 32 mg protein /g of homogenate and during storage at -20°C for 12 months decreased gradually up to fifth month followed by sharp decline and reached to 6.7 at the end of storage period. From the statistical analysis (Table 16), significant differences at $p<0.05$ were found between the second and third month and such trend was more evident between the fifth and eighth month of storage at -20°C. At zero time of storage no significant differences were recorded between lot A and lot B, while significant differences were found

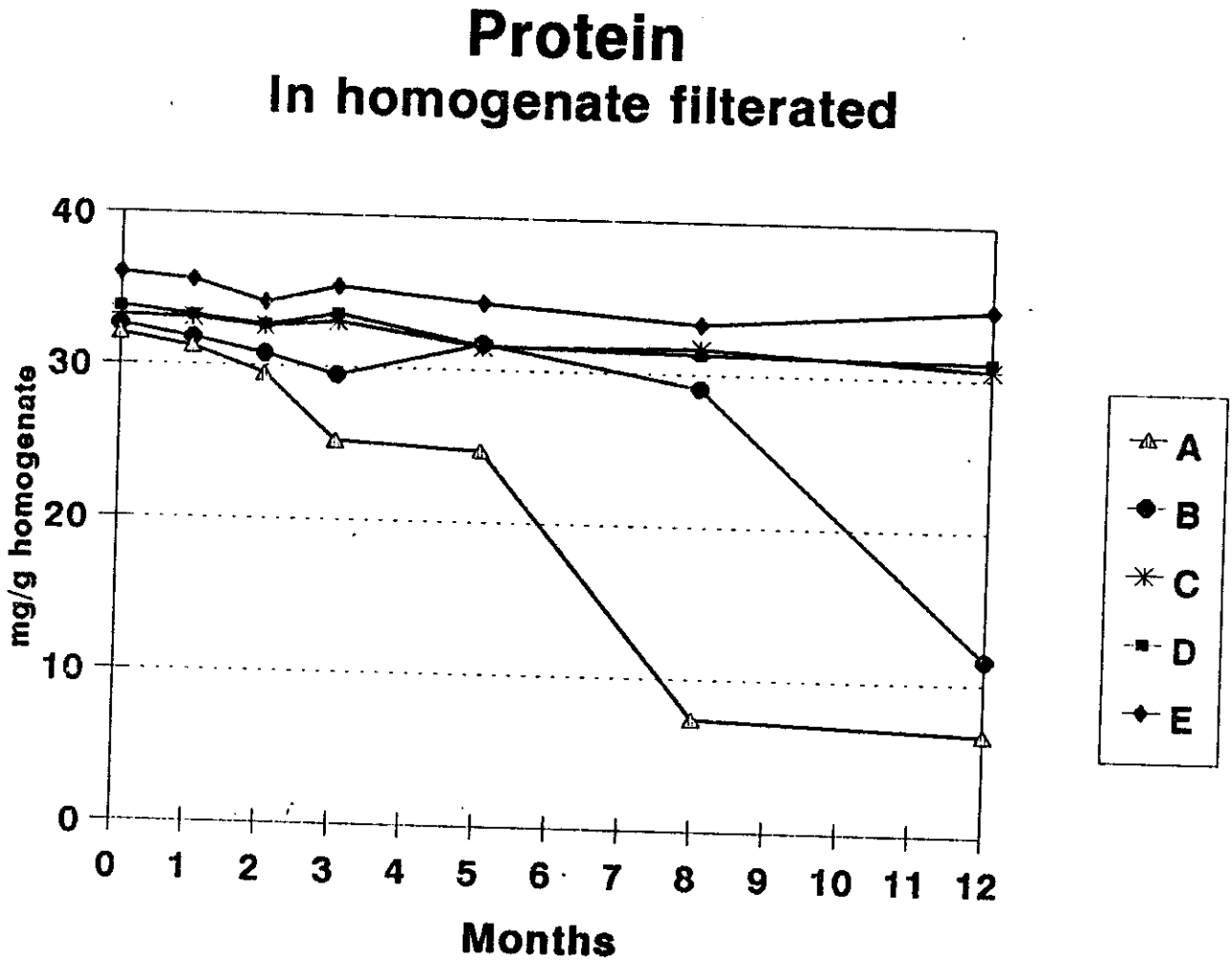
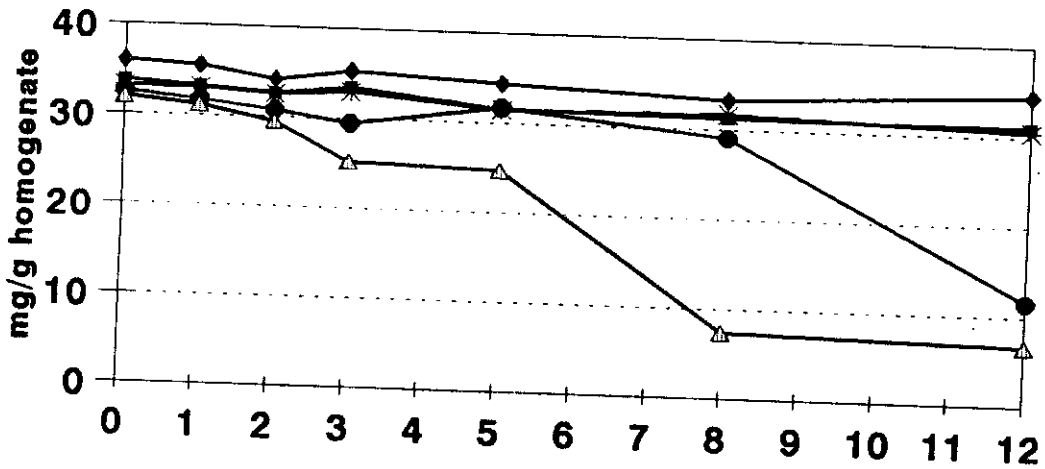


Figure (9). The total protein in the homogenate of different lots during frozen storage at −20°C for one year. Lot A: 100 % mince hake; Lot B: 75 % mince hake + 25 % mince sardine; Lot C: 50 % mince hake + 50 % mince sardine; Lot D: 25 % mince hake + 75 % mince sardine; Lot E: 100 % mince sardine.

Protein
In homogenate filterated



A	32	31.2	29.5	25.2	24.7	7.4	6.7
B	32.6	31.8	30.8	29.5	31.7	29.1	11.6
C	33.2	33.1	32.6	33	31.5	31.7	30.6
D	33.8	33.3	32.7	33.5	31.6	31.3	31.1
E	36	35.6	34.2	35.3	34.4	33.3	34.5

Months

Table (15) Liner regression and analysis of variance of total protein in the homogenate of lots stored at -20°C for 12 months.

X-Y	R GENERAL/α	R_X/α	R_Y/α
A-B	0.8465/0.0000	0.9515/0.0000	0.8411/0.0000
A-C	0.6050/0.0000	0.9515/0.0000	0.8959/0.0000
A-D	0.6541/0.0000	0.9515/0.0000	0.8808/0.0000
A-E	0.5399/0.0003	0.9515/0.0000	0.6028/0.0038
B-C	0.6255/0.0000	0.8411/0.0000	0.8959/0.0000
B-D	0.6255/0.0000	0.8411/0.0000	0.8808/0.0000
B-E	0.5347/0.0003	0.8411/0.0000	0.6028/0.0038
C-D	0.8813/0.0000	0.8959/0.0000	0.8808/0.0000
C-E	0.4501/0.0028	0.8959/0.0000	0.6028/0.0038
D-E	0.4908/0.0010	0.8808/0.0000	0.6028/0.0038

Lot A: 100 % mince hake.

Lot B: 75 % mince hake + 25 % mince sardine.

Lot C: 50 % mince hake + 50 % mince sardine.

Lot D: 25 % mince hake + 75 % mince sardine.

Lot E: 100 % mince sardine.

Table (16) Analysis of variance of total protein in the homogenate for the frozen minced samples during storage at -20°C for 12 months.

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	a/1	ab/1	b/1	c/1	c/1	d/1	d/1
Lot B	a/1	ab/2	bc/1	cd/2	ab/2	d/2	e/2
Lot C	a/2	a/3	ab/3	a/3	cd/2	bc/34	d/3
Lot D	a/3	ab/3	b/3	a/4	c/2	c/3	c/3
Lot E	a/4	a/4	b/4	ac/4	b/3	c/4	b/4

* Different letters in each row indicate significant difference of storage period at ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage.

with other lots. After that at any given time of storage significant differences among lot A and other lots were found.

With respect to lot E; experiments proved the highest value of protein was found in the homogenate of such lot when compared with others and the significant differences were recorded among lot E and the other lots at any given time storage at -20°C. Similar results were given by Huidobro, *et al.* (1992) who showed that the highest value of protein in the homogenate was observed in minced sardine. The total protein in minced sardine changed slightly in the filtrate during storage, where the initial value that was 36 mg/g homogenate decreased to 34.5 mg/g homogenate at the end of storage.

The effect of adding minced sardine to minced hake with different ratios on the total protein of the filtered homogenate is also given in figure (9). In lot B (75 % minced hake + 25 % minced sardine) the initial total protein was similar to lot A, while significant differences were indicated with other lots at any given time of storage at -20°C (Table 16). The initial total protein that was 32.6 mg/g homogenate of lot B changed to 11.6 mg protein/g homogenate at the end of storage at -20°C.

Regarding lot C (50 % hake + 50 % sardine) the initial total protein of the filtrated homogenate was 33.2 mg/g changed slightly to 30.6 mg/g at the end of storage period. From statistical analysis lot C showed significant differences at the begining of storage with others lots, after which no significant differences was found with lot D at any given time of storage, while the significant differences were observed with other lots.

Similar results were noticed in lot D as a result of adding 75 % minced sardine to 25 % minced hake, where the

total initial protein in the filtrated homogenate was 33.8 mg/g homogenate. Such value remained nearly constant up to the third month of storage at -20°C; and the significant differences were only noticed between the third and the fifth month, while no significant differences in the remaining storage period were found. However, the total protein of the homogenate reached to 31.1 mg protein /g homogenate at the end of storage period.

It is worth to note that the total protein in the filtrated homogenate which used to measure apparent viscocity and emulsifying capacity changed slightly during storage of all lots except lot A and B. In lot A the total protein decreased slightly up to the fifth month and then declined sharply up to the end of storage. This may be attributed to the aggregation of proteins which occur during storage at -20°C that became separated when filtering the homogente through the cheese cloth. The obtained results are in agreement with Tejada *et al.* (1984). They showed that the total protein values for homogenate after filtering of bonito, cod and horse mackerel remained constant throughout storage in all species except cod, which showed a remarkable decrease. They explained the latter case by the possibility of harding of the sarcolemma during storage since the muscle fibers were not completely ruptured during blending and retained over cheese cloth during filtration of the homogenate. Careche and Tejada (1990 a) found the same trend where the total protein of homogenate from hake decreased sharply after 245 days of storage at -18°C. Similar behavior has also been described by Huidobro and Tejada (1993) for muscle homogenates of blue whiting, the authors described that the total protein diminished considerably as the aggregates formed in the course of storage period were trapped in the cheesecloth during filtering and reached to 6.56 mg protein per g mince after storage at -18°C for 44 month.

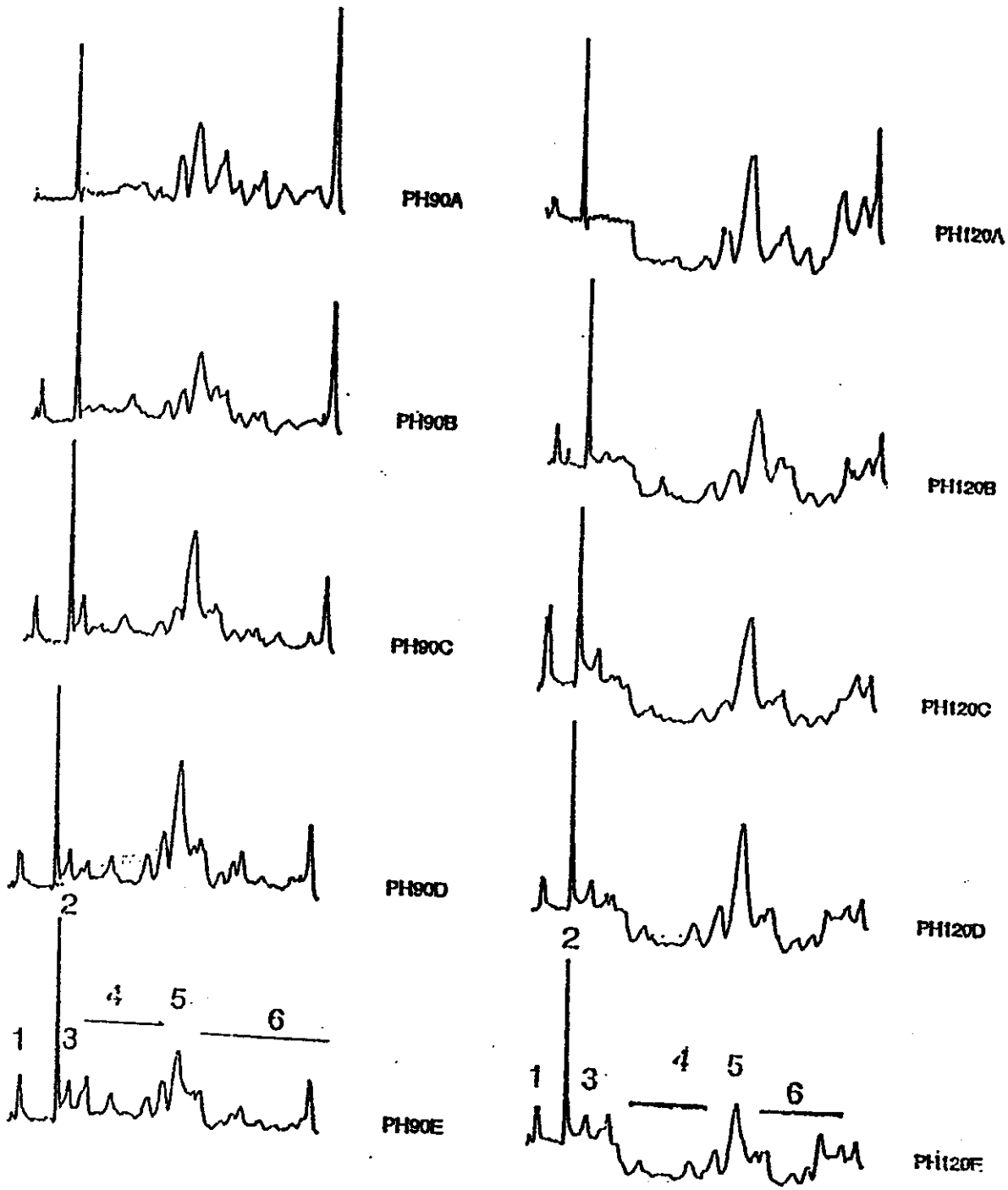


Figure (10) SDS-PAGE of different lots of the filterted protein homogente in 5 % NaCl after 12 months of storage at -20°C. 1. application zone; 2. peak in the stacking/resolving interphase; 3. myosine heavy chain; 4. proteins of MW between 200 and 45 kDa; 5. actin; 6. tropomyosin + troponins + myosin light chains and sarcoplasmic protein.

With respect to lot E the experiments proved that the total protein in the homogenate remained nearly constant throughout storage at -20°C. This may be due to the fact that sardine is more stable during storage under frozen condition and do not form condenced aggregate. Sub_ sequently, no retention of aggregates in the cheescloth was occured and the total protein was obtained in the homogenate.

It is important to notify that when 25 % of minced sardine was added to 75 % minced hake (lot B), the total protein of homogenate increased in relation to lot A; but during frozen storage the total protein was slightly decreased with a lower rate. This may be due to the added 25 % of minced sardine to minced hake decrease the size of the aggregate formed up to the end of storage period. With such view in mind, the total protein was approximately stable during storage by increasing the ratios of minced sardine to 50 % in lot C and to 75 % in lot D.

The examination of electrophoretic patterns of the filterted protein homogenate in 5 % NaCl after 12 months of frozen storage at -20°C of figure 10 showed that in lot A the bands that corresponding to myosin heavy chain (MHC) disappeared from the filtrate while other bands appeared; a trend which indicate the presence of the sarcoplasmic protein. Similar findings were observed in lot B. However in lots C, D and E, MHC was observed .

*** Changes in apparent viscosity:**

The measurement of apparent viscosity is an appropriate method of quality control for myosystems undergoing frozen storage. The relation between storage at -20°C for 12 months and the apparent viscosity in the

muscle homogenates of different minced fish lots is shown in table (18). Table (19) shows the analysis of variance among mean values of apparent viscosity according to storage time and at any given period of storage.

The obtained data (Table 17) proved that the highest values of the correlation coefficient were 0.9353, 0.9181, 0.9124 and 0.9063 for lots A, B, D and E, respectively, while the lowest value was for lot C (R = 0.7646). Signi_ ficant differences were found within the different lots under similar conditions of storage.

Comparing the initial values of apparent viscosity for investigated samples (Table 19), a significant difference was found among different lots and such trend was more pronounced in most period of storage at -20°C for 12 months. The highest initial value of apparent viscosity was noticed for lot A. During storage at -20°C the apparent viscosity decreased sharply up to the second month; followed by a gradual decrease until the eighth month, after which changes in viscosity were very limited to a degree that do not register on the measurement scale under the experimental conditions. With statistical view points; table 19 proved the presence of significant differences up to the thrid month, while no significant differences were noticed up to the end of storage period.

The apparent viscosity of minced sardine (lot E) was initially the lowest one when compared with other lots. However, under frozen storage and up to 12 months the apparent viscosity decreased with a lower rate, since, it regists the highest value from the second month. Statis_ tical data in table 19 indicated that there were significant differences were observed from zero-time up to the end of storage at -20°C, and also significantly different was found among lot E and other lots at any given time of storage.

Table (17) Liner regression and analysis of variance of apperant viscosity of lots stored at -20°C for 12 months.

X-Y	R GENERAL/α	R_X/α	R_Y/α
A-B	0.9256/0.0000	0.9353/0.0000	0.9181/0.0000
A-C	0.8222/0.0000	0.9353/0.0000	0.7646/0.0001
A-D	0.8350/0.0000	0.9353/0.0000	0.9124/0.0000
A-E	0.6356/0.0000	0.9353/0.0000	0.9063/0.0000
B-C	0.8127/0.0000	0.9181/0.0000	0.7646/0.0001
B-D	0.8250/0.0000	0.9181/0.0000	0.9124/0.0000
B-E	0.6024/0.0000	0.9181/0.0000	0.9063/0.0000
C-D	0.8221/0.0000	0.7646/0.0001	0.9124/0.0000
C-E	0.5487/0.0002	0.7646/0.0001	0.9063/0.0000
D-E	0.6666/0.0000	0.9124/0.0000	0.9063/0.0000

Lot A: 100 % mince hake.

Lot B: 75 % mince hake + 25 % mince sardine.

Lot C: 50 % mince hake + 50 % mince sardine.

Lot D: 25 % mince hake + 75 % mince sardine.

Lot E: 100 % mince sardine.

Table (18) Changes in Apparent viscosity (cP) of different lots during frozen storage at -20°C for 12 months.

	Storage period at -20°C						
Months	0	1	2	3	5	8	12
Lot A	20200	5983	286.5	66.5	60	20.67	ND
Lot B	12541	2803	165.5	109.5	70.16	61.5	ND
Lot C	9533	3310	313.17	274.7	127.7	122.2	108.3
Lot D	8500	3530	1015.8	1093	212.8	168.7	117.5
Lot E	8133	3913	3463.3	2657	2657	2610	1376.7

ND = not detected

Lot A: 100 % mince hake.

Lot B: 75 % mince hake + 25 % mince sardine.

Lot C: 50 % mince hake + 50 % mince sardine

Lot D: 25 % mince hake + 75 % mince sardine

Lot E: 100 % mince sardine.

Table (19) Analysis of variance of apparent viscosity for the frozen minced samples during storage at -20°C for 12 months.

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	a/1	b/1	c/1	d/1	d/1	d/1	d/1
Lot B	a/2	b/2	c/2	d/1	e/1	e/2	f/1
Lot C	a/3	b/3	c/1	c/2	c/12	c/3	c/2
Lot D	a/4	b/4	c/3	c/3	d/2	de/4	e/2
Lot E	a/5	b/5	c/4	d/4	e/3	e/5	f/3

* Different letters in each row indicate significant difference of storage period at ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage.

With respect to lot B, adding 25 % of minced sardine to 75 % minced hake the initial value of apparent viscosity was reduced when compared with lot A, since the value was intermediate between lots A and E. During storage at -20°C decline of apparent viscosity was rapid up to the second month while at the end of storage period value of apparent viscosity was out detection under experimental conditions. From the analysis of variance, lot B was signficantly different with other lots up to the second month of storage at -20°C.

In lot C when adding 50 % of minced sardine to 50 % of minced hake, the values changed from 9533 cP at the begining of storage to 108.33 cP at the end of storage period. Significant differences ($p<0.05$) were found from zero-time until the second month after which no significant differences were noticed up to the remainder of storage period at -20°C. Similar trends were noticed when adding three parts of minced sardine to one part of minced hake (lot D).

The obtained results indicate a real inverse relation between apparent viscosity and storage period at -20°C. Such trend could be due to denaturation and protein aggre_ gation during frozen storage which lead to decrease of protein solubility. Such trend was more evident in lot A. The previous results are in agreement with that of Jiménez-Colmenero and Borderias (1983) who showed that frozen storage lowered the viscosity of the protein extracts and viscosity depends on soluble protein concentration, which decrease throughout the frozen storage period as a result of protein denaturation and aggregation. Similar results were given by Careche and Tejada (1990 a).

The differences in initial apparent viscosity between lot A (lean fish) and lot E (fatty fish) may be due to the presence of fat in the homogenate of minced sardine that

decreased the vailability of hydrophilic groups, and so reduced the apparent viscosity (Jiménez-Colmenero *et al.* 1988). The decrease in apparent viscosity in lot E at lower rate in relation to lot A may be due to lower decrease of protein solubility of lot E during frozen storage as shown in figure (8). Such trend was previously reported by Tejada *et al.* (1984), who showed that the decrease in viscosity in the homogenate in 5 % NaCl from bonito and horse mackerel was lower over the storage period than it was in cod due to the slight decrement in protein solubility levels. Same findings were also mentioned by Jiménez-Colmenero *et al.* (1988). They showed that significant differences were observed in viscosity and protein solubility during storage in hake muscle, while the changes in the apperent viscosity were less evident in sardine.

With respect to the changes in viscosity of minced hake as affected by addition of minced sardine, from the obtained results it can be seen that the increase ratio of minced sardine caused a reduction in the initial value of apparent viscosity, in spite of the decrement which occured during frozen storage the samples showed higher values. Careche and Tejada (1990 a) also showed that the apparent viscosity of the control sample (minced hake) decreases sharply during the first month of storage until reaching, after 3 months, lower values which do not measured under the same condition. However, when neutral and oxidized lipids were added, the sample with oxidized lipids had higher values than the control although the decrease in viscosity was also sharp.

From the above results, it could be concluded that the rate of reduction in apparent viscosity with time was faster for minced hake than other lots. Lot E showed the lower decrease in viscosity and variation in the ratio of minced sardine which added to minced hake may lead to differences in the rate of decrease in viscosity. The

present results which demonstrates a decrease in apparent viscosity during frozen storage, indicate the presence of denaturation of protein with a reduction in salt soluble protein (myofibrillar protein). Borderias *et al.* (1985 b) stated that the myofibrillar protein was the main respon_ sible for the viscosity of the homogenates and the lower apparent viscosity of the fish muscle extracts may be the result of greater lability of myofibrillar proteins.

In order to determine whether the variations in apparent viscosity of different lots corresponds with a difference in the behaviour of the myofibrillar proteins during frozen storage, SDS-polyacrylamide gel electro_ phoresis was performed on the homogenate at the end of the storage period for minces fish as seen in figure (10). The results of the electrophoretic diagrams for lot A did not include myosin heavy chain (MHC), on contrary in lot E the bands appeared which indicating that MHC of sardine was more stable during storage when compared with lot A. In lot B (which contain 25 % of minced sardine) the MHC became weaker, and by increaseing the ratio of minced sardine which added to minced hake as in lot C and D MHC was checked out. These results indicate the relationship between the myofibrillar proteins (mainly MHC) and apparent viscosity. Tejada *et al.* (1984) reported that the apparent viscosity of the muscle homogenates was basically due to the myofibrillar protein.

*** Changes in emulsifying capacity:**

The emulsifying capacity (EC) of different lots of minced fish was ploted in figure (11) against storage for 12 months at -20°C. The statistical analysis are shown in tables (20 and 21).

Results of the regression analysis (Table 20) showed that the highest correlation coefficient (R) was recorded

Table (20) Liner regression and analysis of variance of emulsion capacity of lots stored at -20°C for 12 months.

X-Y	R GENERAL/α	R_X/α	R_Y/α
A-B	0.8226/0.0000	0.9334/0.0000	0.7665/0.0001
A-C	0.7789/0.0000	0.9334/0.0000	0.7895/0.0000
A-D	0.7133/0.0000	0.9334/0.0000	0.8291/0.0000
A-E	0.5722/0.0001	0.9334/0.0000	0.7561/0.0001
B-C	0.7591/0.0000	0.7665/0.0001	0.7895/0.0000
B-D	0.7134/0.0000	0.7665/0.0001	0.8291/0.0000
B-E	0.5487/0.0002	0.7665/0.0001	0.7561/0.0000
C-D	0.7753/0.0000	0.7895/0.0000	0.8291/0.0000
C-E	0.5751/0.0001	0.7895/0.0000	0.7561/0.0001
D-E	0.6384/0.0000	0.8291/0.0000	0.7561/0.0001

Lot A: 100 % mince hake.

Lot B: 75 % mince hake + 25 % mince sardine.

Lot C: 50 % mince hake + 50 % mince sardine.

Lot D: 25 % mince hake + 75 % mince sardine.

Lot E: 100 % mince sardine.

for lot A (R = 0.9334) followed by lot D (R = 0.8291), while that of other lots were seemed to be similar. The ANOVA analysis of the same table showed the presence of a significant differences among different lots at ($p<0.05$).

From the obtained results, minced hake initially showed the lowest value of emulsifying capacity and no significant differences were found among lots B, C and D. The differences in the EC of the species could be due to presence of lipids, since species that displaying the highest EC, is the one with the highest fat content, a trend which was pronounced in lot E rather than minced hake (lot A) which is actually a lean species. Huidobro and Tejada (1993) stated that in fish, initial EC varies according to species, but in general EC declines in the course of frozen storage, essentially in species forming dimethylamine and formaldehyde. However, the EC was reversibly correlated with frozen storage, a pattern which was more pronounced in minced hake; where the initial EC value was 38.19 and decrease gradually up to the thrid month followed by a sharp fall in the 8 th month up to reach lower value (23.42) at the end of storage period. Similar trend was noticed by Careche and Tejada (1990 a); and Srikar and Reddy (1991) who stated that the emulsifying capacity of minced pink perch (*Nemipterus japonicus*) show a significant decrease in the efficiency of minced fish to emulsify oil during frozen storage. Grabowska and Sikorski (1974) observed also a similar phenomenon in cod minces and found that EC, which were significantly correlated (0.85-0.95), decreased due to denaturation and protein aggre_ gation induced by frozen storage.

The available data also proved that the higher EC was recorded in minced sardine. The EC was 49.54 at zero-time of storage and reached to 35.96 at the end of storage period. So, it could be concluded that EC in lot E was reduced during frozen storage, while it maintained the

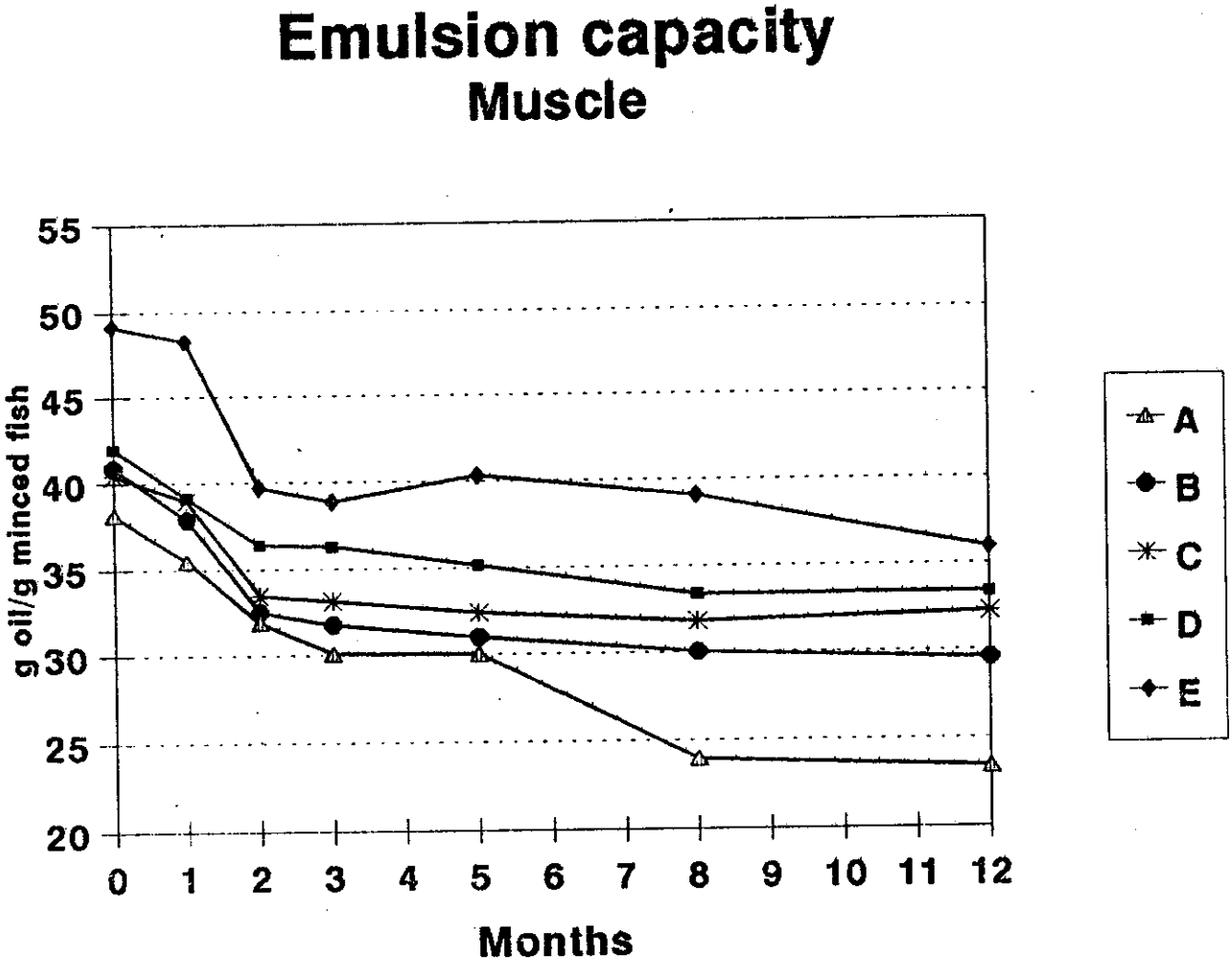
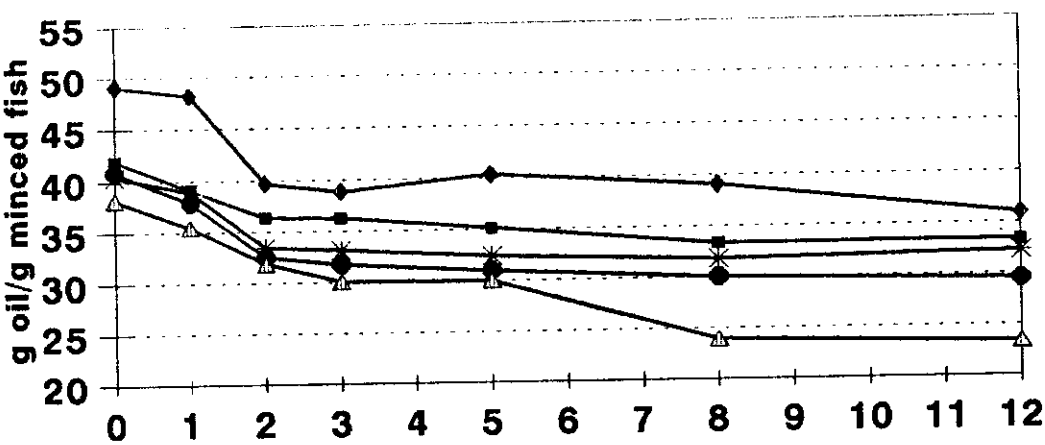


Figure (11). Changes in emulsifying capacity of different lots during frozen storage at -20°C for 12 month. Lot A: 100 % mince hake; Lot B 75 % mince hake + 25 % mince sardine; Lot C 50 % mince hake + 50 % mince sardine; lot D: 25 % mince hake + 75 % mince sardine; Lot E: 100 % mince sardine.

Emulsion capacity
Muscle



A	38.19	35.48	31.92	30.09	30.03	23.96	23.42
B	40.88	37.9	32.53	31.78	31.01	30.06	29.59
C	40.43	38.96	33.48	33.15	32.45	31.82	32.27
D	41.99	39.14	36.4	36.31	35.15	33.39	33.4
E	49.13	48.24	39.7	38.88	40.33	39.08	35.96

Months

Table (21) Analysis of variance of emulsion capacity for the frozen minced samples during storage at -20°C for 12 months.

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	a/1	b/1	c/1	c/1	c/1	d/1	d/1
Lot B	a/2	b/2	c/12	cd/1	de/2	de/2	e/2
Lot C	a/12	a/2	b/2	bc/2	cd/2	cd/23	d/3
Lot D	a/2	b/2	c/3	c/3	cd/3	d/3	d/4
Lot E	a/3	a/3	b/4	b/4	b/4	b/4	b/5

* Different letters in each row indicate significant difference of storage period at ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage.

largest value of emulsified oil per g mince when compared with other lots. However, significant differences were found with other lots at any given time of storage. Tejada *et al.* (1984) studied the effect of frozen storage on emulsifying capacity for bonito, cod, horse mackerel and chicken muscle. They showed that EC decreased over storage for both total protein extract and salt soluble protein fractions, this drop being most apparent in cod muscle.

The changes in emulsifying capacity as a results of adding different ratios of minced sardine to minced hake are given in the same figure and tables. The addition of 25 % of minced sardine (lot B) increased the initial value of EC when compared with the corresponding value of lot A. A severe reduction in EC occurred during storage at -20°C up to the second month followed by a slight decrease within the remainder of the storage period, although this reduction in EC of lot B displayed higher values in relation to minced hake.

With respect to lots C and D there is no evidence of changes in the initial value of EC in relation to lot B. The available results reflects also the same reduction trend of the values during frozen at -20°C for 12 months, but the EC values at any given time of storage was higher than the corresponding values in lots B and A. The obtained results are in agreement with Careche and Tejada (1990 a) who studied the effect of added neutral and oxidized lipids on the emulsifying capacity of minced hake during frozen storage. They noticed that the greatest percentage of variations occurs in the first two months of storage. Although the three samples started with similar EC values; the sample with oxidized lipids showed values which were significantly higher than the control throughout the storage period, while the sample with neutral lipids having values slightly higher than those of the control sample.

From the aformentioned results it could be conculded that values of EC decreased progressively in all the studied samples as affected by frozen storage at -20°C due to the changes in the solubility (fig. 8) and the amount of protein in the homogenate (fig. 9). The loss in EC values was more pronounced in minced hake (lot A) from 8th month due to lower solubility and less protein in the homogent than that recorded for minced sardine. On the other hand, adding minced sardine to minced hake reduced the loss in EC value as showen in lot B, C, and D. It is important to notify that the concurrent decrease in muscle protein solubility figure (8) as a result of denaturation induced by frozen storage may explain the reduction in emulsifying capacity, since a positive correlation between protein solubility and emulsifying capacity has been reported by Volkert and Klein (1979). Srikar and Reddy (1991) stated that there are many factors affecting the relationship between EC and Protein solubility, among these factors is the salt soluble protein (SSP) concentration which decreased throughout the frozen storage period ($p<0.05$) as a result of protein denaturation and aggregation leading to a decrease in EC during frozen storage. Hence, the higher level of SSP in the mince, the higher is the EC of the muscle proteins; as the EC technique is accomplished by sodium chlorid extraction it is postulated that only SSP were effective in the emulsification of oil. Borderias *et al.* (1985 a) also found that variations in the concen_ tration of myofibrillar proteins could be one of the main factors affecting the emulsifying ability.

Through examination of the relationship between myofibrillar proteins and EC, the electrophoretic patterns of the investigated filtered protein homogenate in 5 % NaCl after 12 months at -20°C of frozen minces fish (figure 10) showed the bands corresponding to the myosin heavy chains (MHC) of lot (A) disappear while other bands corresponding to lower molecular weight appeared. Such trend indicates

the presence the sarcoplasmic protein which play part in the formation of emulsions. Such trend was observed in lot B. In relation to lot E the bands which indicate the presence of MHC appeared, so in lot E, values of EC were higher than lot A. Similar trend was found in lots C and D.

*** Changes in emulsion stability:**

Proteins are used as stabilizers in emulsions since they tend to adsorb at the oil-water interface, thereby modifying interparticle forces, and providing kinetic stability to emulsion, due to the presence of hydrophobic and hydrophilic functional groups, the former one is usually point into the oil phase whereas the latter one is exposed to the aqueous medium. Consequently, in oil-in-water emulsion, the hydrophobic functional groups adsorb at the oil droplet surface in the form of trains with protruding of hydrophilic functional groups into the aqueous medium in the form of loops and trains (Narsimhan, 1992).

Figure (12) shows the variations in emulsion stability of the filtred homogenate of different lots during frozen storage and table (22) shows the analysis of variance of different lots during frozen storage at -20°C for 12 months.

The available data proved that lot A (100 % minced hake) had the initial percentage of total exudate 30.65 % of which 29.37 % water and 1.28 % fat. During storage at -20°C the emulsion stability decreased, due mainly to the fact that the amount of oil separation increased. Such trend was more pronounced at the eighth months, where total exudate reached to 89.04 % of which 36.75 % was water and 52.30 % was separated oil. The increase of total exudate was due to a big increase of oil released. After 12 month of storage at -20°C the emulsion at 80 % maximum oil was

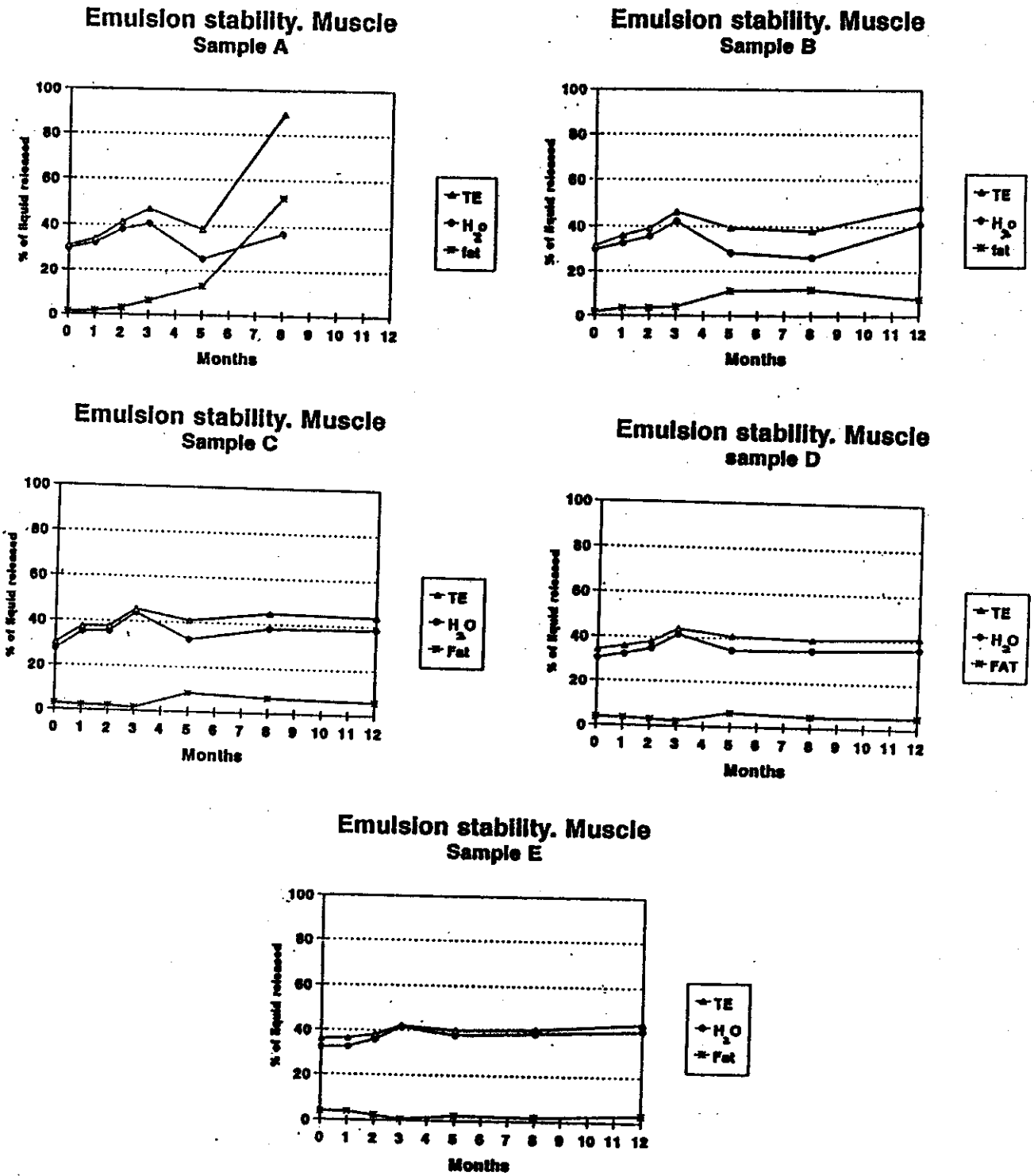
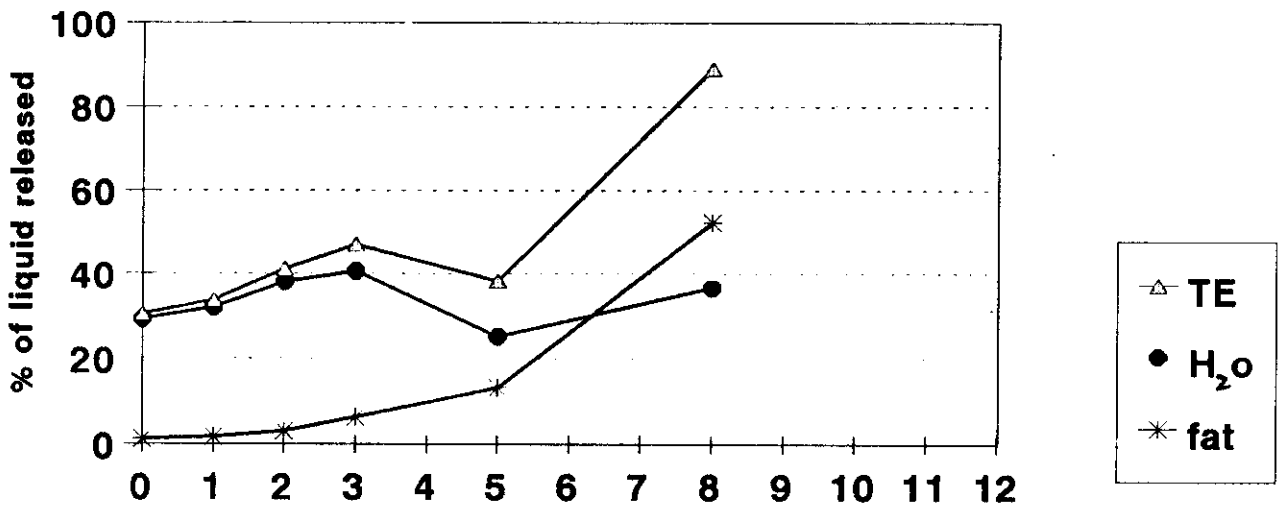


Figure (12). Changes in emulsion stability of muscles of different lots during frozen storage at -20°C for 12 month. Lot A: 100 % mince hake; Lot B: 75 % mince hake + 25 % mince sardine; Lot C: 50 % mince hake + 50 % mince sardine; Lot D: 25 % mince hake + 75 % mince sardine; Lot E: 100 % mince sardine.

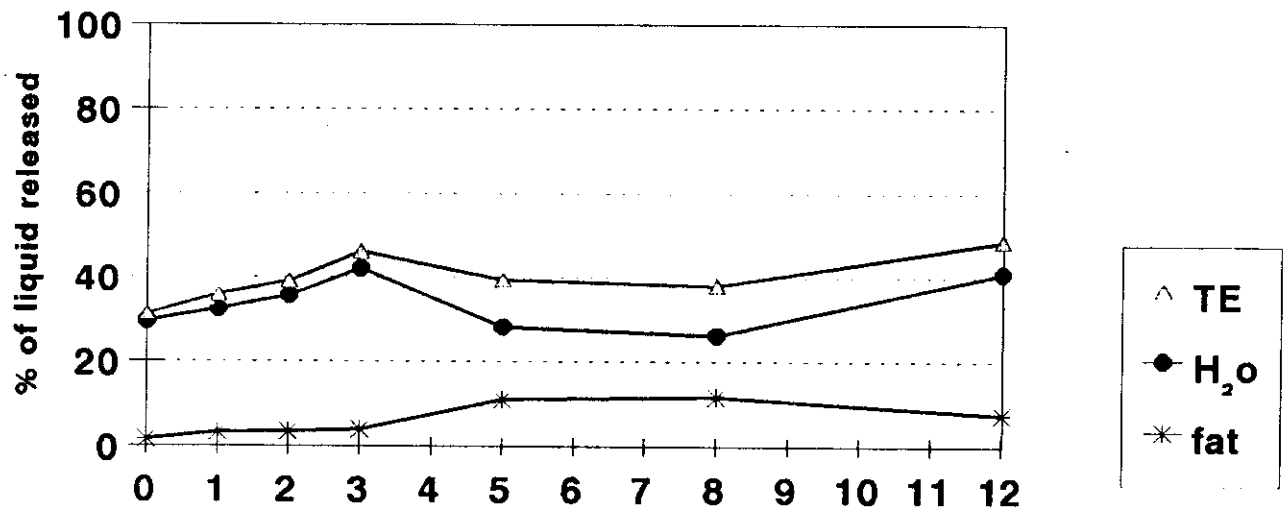
Emulsion stability. Muscle
Sample A



TE	30.65	33.88	41.35	47.14	38.39	89.04
H_2o	29.37	32.15	38.19	40.71	25.27	36.75
fat	1.28	1.73	3.16	6.43	13.12	52.3

Months

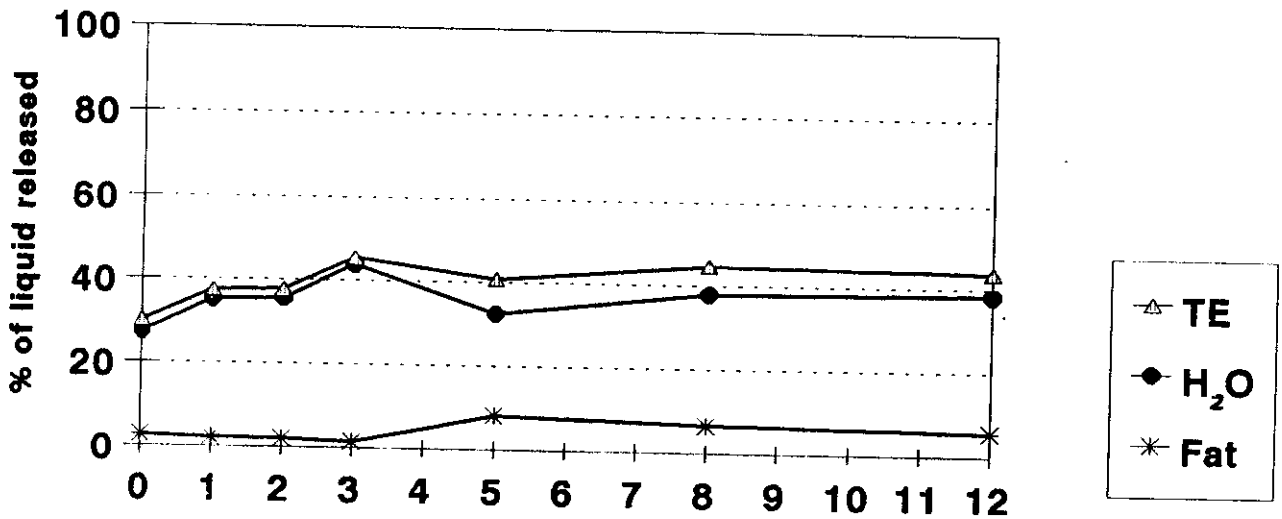
Emulsion stability. Muscle
Sample B



TE	31.25	35.9	39.2	46.18	39.4	38.08	48.73
H_2o	29.6	32.47	35.68	42.15	28.23	26.24	41.06
fat	1.65	3.43	3.52	4.03	11.17	11.84	7.66

Months

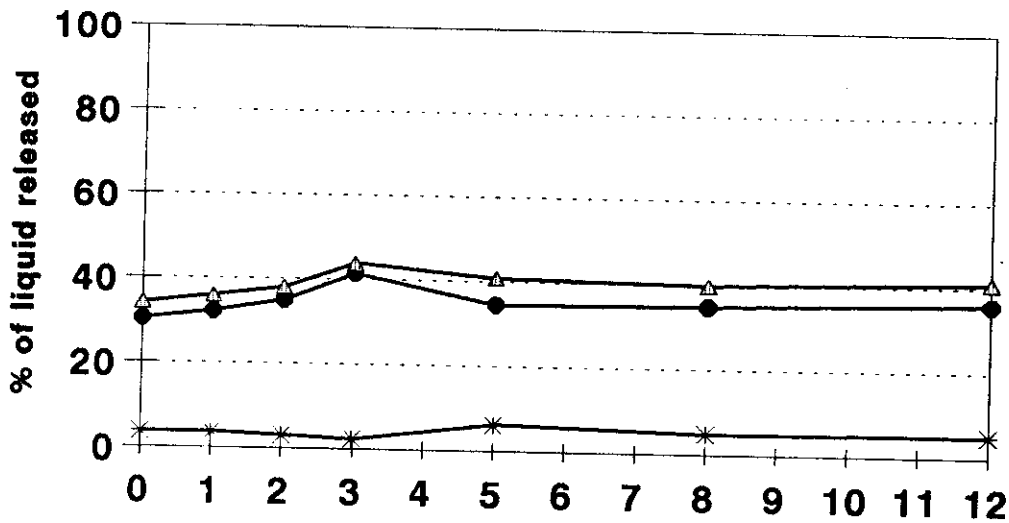
Emulsion stability. Muscle
Sample C



TE	30.22	37.57	37.78	45.53	40.88	44.73	43.85
H_2O	27.39	35.25	35.62	43.93	32.58	37.99	38.4
Fat	2.83	2.32	2.16	1.6	8.3	6.74	5.81

Months

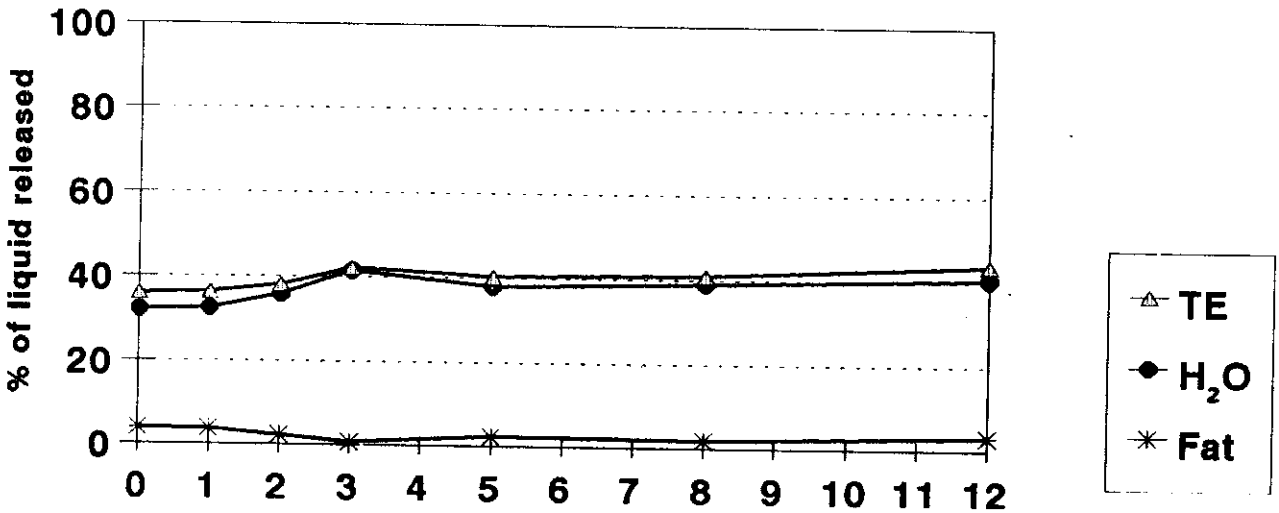
Emulsion stability. Muscle
sample D



TE	34.2	36.01	38.11	43.9	40.85	39.89	41.1
H_2O	30.45	32.31	35.01	41.47	34.62	34.94	36.05
FAT	3.75	3.72	3.1	2.43	6.25	4.96	5.05

Months

Emulsion stability. Muscle
Sample E



TE	36.06	36.38	38.19	42.28	40.42	41.02	43.85
H_2O	32.13	32.55	35.88	41.54	38.04	39.08	40.68
Fat	3.93	3.83	2.31	0.74	2.38	1.94	3.17

Months

Table (22) Analysis of variance of emulsion stability for frozen minced fish stored at -20°C for 12 months.

Total exudate

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	a/1	ab/1	cd/1	c/1	bd/1	e/1	---
Lot B	a/12	b/12	c/12	c/1	b/12	b/2	c/1
Lot C	a/1	b/2	b/2	c/1	bd/23	c/3	cd/2
Lot D	a/12	b/12	c/2	d/1	ef/23	e/24	f/3
Lot E	a/2	a/12	ab/2	cd/1	bc/13	bc/4	d/2

Water released

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	ab/1	ac/1	cd/1	d/1	a/1	bcd/1	---
Lot B	ab/1	ac/1	c/12	d/1	b/2	b/2	d/1
Lot C	a/1	bc/2	bc/12	d/1	b/3	c/1	c/2
Lot D	a/1	b/1	cd/2	e/1	c/3	cd/1	d/3
Lot E	a/1	a/1	b/12	c/1	bd/4	cd/1	cd/1

Fat separation

	Storage period at -20°C						
Mon.	0	1	2	3	5	8	12
Lot A	a/1	a/1	ab/1	b/1	c/1	d/1	---
Lot B	a/1	b/2	b/1	b/2	c/2	c/2	d/1
Lot C	a/2	ab/1	ab/2	b/3	c/3	d/3	e/2
Lot D	a/3	b/2	ab/1	b/3	c/4	d/34	d/2
Lot E	a/3	a/2	bc/2	d/4	b/5	c/4	e/3

* Different letters in each row indicate significant difference of storage period at ($P<0.05$) for each lot.

* Different numbers in each column indicate significant differences between lots at ($P<0.05$) for each period of storage.

broken. Statistical analysis given in table (22) indicated significant differences of total exudate between the first and second month and more evident between the fifth and eighth month. At the begining of storage significant differences of total exudate were observed between lot A and lot E, while no significant differences were found with the other lots. In the eighth month significant differences were showed among lot A and the other lots.

Regarding lot E (100 % minced sardine) (figure 12), higher values of total exudate (36.06 %) of which water released was 32.13 % and fat separation was 3.93 % were noticed at the begining of storage. On the other hand, the emulsions in lot E were more stable than in lot A. Lot E showing a slight increase with time of all parameters. At the end of storage period total exudate reached 43.85 % and water released 40.68 %. Since lot E is very stable in fat released, the slight an increase of total exudate was found due mainly to water release.

From the results it could be noticed that in lot B (75 % minced hake + 25 % minced sardine) the initial parameters which indicate the stability of the emulsion were: total exudate (31.25 %), of which water (29.60 %) and oil separation (1.65 %). These values changed to 48.73 %, 41.06 % and 7.66 %, respectively at the end of storage period. At the begining of storage no significant differences of all parameters were found between lot B and lot A.

In case of lot C (50 % minced hake + 50 % minced sardine), the initial total exudate was similar with lot A; since the exudate value was 30.22 %. A noticeable increment in total exudate with advancing the storage period up to the third month after which the values remained constant reaching 43.85 % at the end of storage period. From the statistical analysis no significant differences were found

within oil released until the third month, while the significant differences were recorded in the remaining of the storage period.

Similar results were observed for lot D (25 % minced hake + 75 % minced sardine) where the initial total exudate (34.20 %), which contain (30.45 % water and 3.75 % oil separation). The total exudate increased during frozen storage and reached to 43.90 % with enhancement of water separation and the values of oil separation remained steady. The statistical analysis indicated a significant differences for the total exudate and water released during frozen storage, while for the oil separation no significant differences were found .

Many proteins, including fish proteins are used as emulsifiers, which lowering the interfacial tension and being strongly adsorbed in the fat-water interface by the orientation of polar groups towards the water and the nonpolar groups towards the fat. The fat particles are surrounded by a film of protein at least a monolayer, giving them a mechanical protection against coalescence. Subsequently, it is clearly that protein membrane should have the capability of stabilizing the emulsion.

Results showed that initially in lot E the emulsion was less stable when compared with lot A, where lot E the highest fat content displaying the highest percentage of total exudate due mainly to fat released. The differences in the emulsion among species may be due to the presence of muscle lipids. This was more evident when added higher proportion of minced sardine to minced hake (lot D). Also when added 25 % minced sardine (lot B) and 50 % (lot C) were followed by small higher percentage of oil separation when compared with lot A (100 % minced hake).

From the previous results, it could be noticed that on prolonging the frozen storage period of different lots caused lower thermal emulsion stability (total exudate and fat and water released) with different ratios within the investigated lots. This reduction was subjected to the result of declining protein functional quality caused by increased denaturation and aggregation with advancing the storage period (as described by Jiménez-Colmenero, *et al.* 1995).

With respect to lot A the results showed a gradually decrease in emulsion stability during frozen storage, while in the eighth month the emulsion was less stable as indicated by much increase in total exudate, water and oil separation. These changes coinciding with the reduction in the emulsion capacity (figure 11) during frozen storage at -20°C for 12 month, as well as with a change in the rate of loss of protein solubility. However, the reduction in the emulsion stability may be due to the changes occured in proteins of minced hake during frozen storage which makes the insoluble protein and muscle fibers become harder and trapped in the cheescloth during the filtration of the homogenates. Subsequently the quantity of proteins present was not adequate to stabilize the emulsion, where the protein-lipid membranes around the globules of fat is weak and rupture to a degree that permits the liquid oil flows out and spreads. At the end of storage period, the emulsion was not formed at 80 % oil, a pattern which attributed to the lower concentration of protein which was not enough to surround the lipid droplets by a protein coating. This was more evident when determining the total protein in the filtrated homogenate (figure 9) which decreased through storage at -20°C and reached slightly amount at the end of storage.

In lot E the emulsion was more stable during frozen storage when compared with lot A, this may be attributed to the high concentration of protein in its homogenate figure (9) and the slight changes in protein solubility as shown in figure (8). Pearson *et al.* (1965) reported that only the fraction of protein which is soluble can function as an effective emulsifying agent. Moreover, Franzen and Kinsella (1976) suggested that, as a protein becomes more soluble, it forms layers around the fat droplet to facilitate asso_ ciation with the aqueous phase, where soluble proteins enclose the fat globules and render the emulsion more stable to heat treatment.

Regarding lot B it was observed that addition of 25 % of minced sardine showed that the emulsion stability was nearly similar up to the fifth month when compared with lot A, while in the eighth month emulsion stability of lot B was more stable than lot A and the emulsion can be made at the end of storage period at 80 % oil. This is probably due to the addition of 25 % of minced sardine which lead to the increment of total protein in the homogenate when compared with lot A. Such conclusion could be supported by the observation of the emulsion capacity figure (11).

With respect to lots C and D, the results showed that both lots displayed similar results with slight differences in the parameters related to the stability of the emulsion, but the oil separation during frozen storage is lower in both lots when compared with lot B. This may be due to that the increment of the added ratios of minced sardine to minced hake increased the total protein in the homogenate with slight changes during frozen storage figure (9). In addition the proteins of minced sardine were more stable than proteins of minced hake.

Part III: Effect of frozen storage at -20°C on the extractability of natural actomyosin (NAM):-

A.1. Extractability of natural actomyosin (S1 Fraction):-

Apparently, the changes of fish muscle proteins during frozen storage is mainly due to the alteration of myofibrillar proteins through denaturation and aggregation. These changes in protein was evaluted by measuring the extractability of natural actomyosin during frozen storage. The changes in the amount of actomyosin extractable in 0.6 M sodium chloride from minces which had been stored frozen for 12 months at -20°C are given in figure (13).

The initial amount of actomyosin extractable of lot A (100 % minced hake) after freezing was 357.2 mg/g protein. After one month of storage at -20°C the amount decreased rapidly followed by a continuous gradually decline up to the third month. After fifth month of storage no extraction of natural actomyosin from minced hake was obtained. Jarenbäck and Liljemark (1975) showed that the amount of protein in myofibrillar extracts obtained from cod stored at -10°C decreased rapidly and reached a minimum after 30 weeks and a less rapid decrease was found at -20°C.

The highest amount of actomyosin extractable was found in lot E (100 % minced sardine) when compared with other lots, where the initial amount of extraction of actomyosin was 431.3 mg/g protein. A gradual decrease in extraction of actomyosin was observed by prolonging the storage period to 12 months at -20°C, and reached to 173.7 mg/g protein at the end of storage period. From the obtained results it could be noticed that the amount of extractable actomyosin of lot E at any given time of storage at -20°C is the highest.

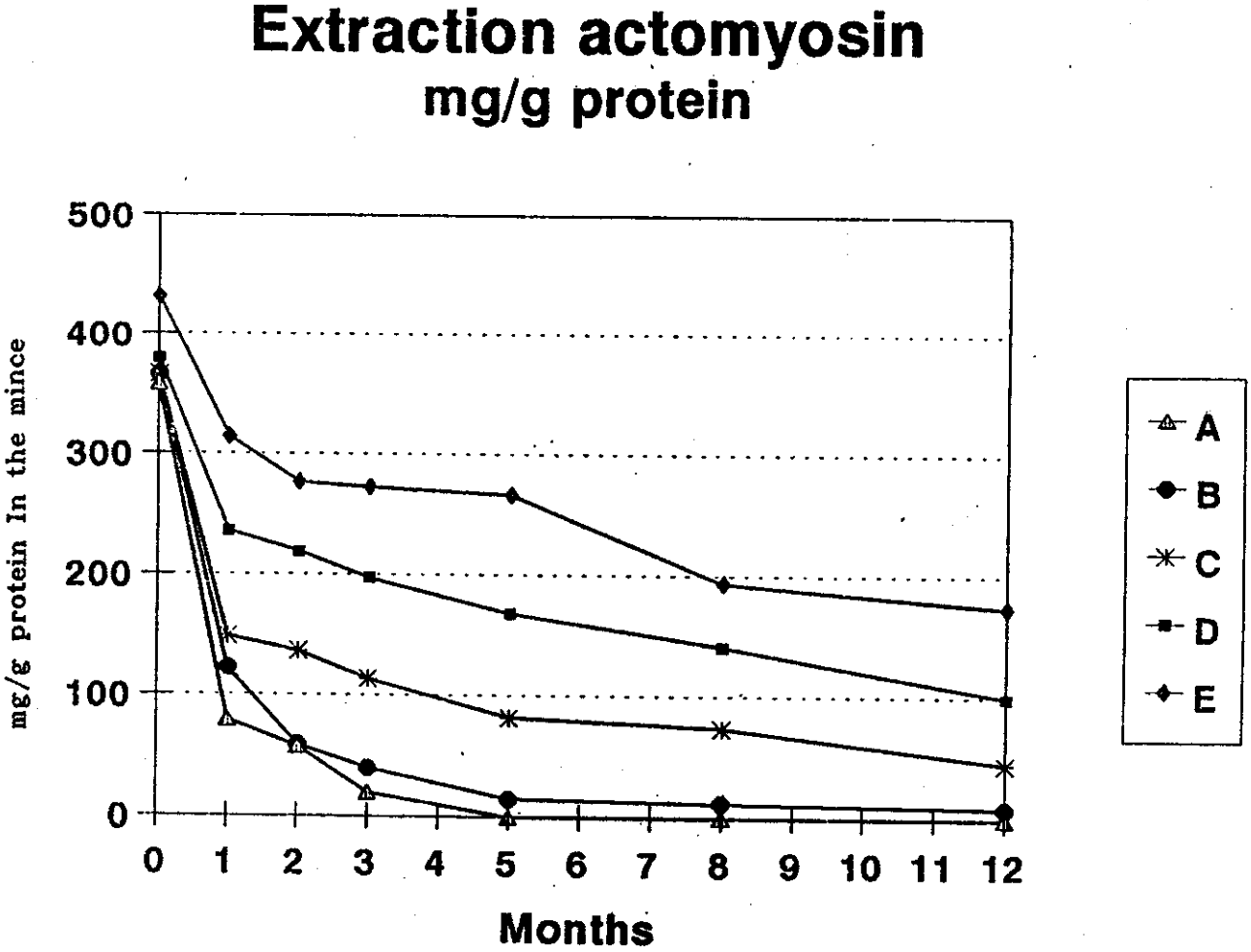


Figure (13). Changes in extraction of actomyosin of different lots during frozen storage at -20°C for 12 months. Lot A: 100 % mince hake; Lot B: 75 % mince hake + 25 % mince sardine; Lot C: 50 % mince hake + 50 % mince sardine; lot D: 25 % mince hake + 75 % mince sardine; Lot E: 100 % mince sardine.

Changes in extractability of actomyosin in minced hake as affected by addition different ratios of minced sardine during frozen storage at -20°C for 12 months were given in the same figure. From the results it was observed that in lot B (75 % minced hake: 25 % minced sardine) had a slight increase in extractable actomyosin in relation to lot A, where the initial value was 365.4 mg/g protein. During frozen storage a sharp decrease in extractability of actomyosin was shown after the first month followed by a gradual decrease and reaching the minimum extraction (8.7 mg/g protein in the mince) after 12 months of storage at -20°C. Similar trend was shown in lot C, in spite of increased the amount of minced sardine in relation to minced hake (50 % minced hake: 50 % minced sardine), the results exhibited no evidence of changes in the initial value when compared with lot B. The extractibility of actomyosin of lot C droped noticeanly from 365.2, the initial value to 148.2 mg/g protein after 30 days of storage and a cotinuous decrement was recorded with advancing of storage period where, the amount of extractable actomyosin reached to 44.6 mg/g protein at the end of storage period. This lot, which contained 50 % of minced sardine had higher levels of extractable actomyosin during frozen storage than lot B.

Regarding lot D which contained 75 % amount of minced sardine the initial amount of actomyosin extractable after freezing was 375.8 mg/g protein. The obtained results indicated that reduced the amount of actomyosin extra_ctable with the length of frozen storage, where the actomyosine extractable had fallent to 99.5 mg/g protein after 12 months at -20°C. In spite of a gradual decrease in actomyosin extractable, the rate of decrease was lower when compared with lots B and C.

During frozen storage in each sample the extrac_ tibility of actomyosin in 0.6 M NaCl decreased indicating that denaturation and aggregation occurs. Similar results were shown by Jiang and Lee (1985) and Jiang, *et al* (1987). The decrease in extractibility indicated that inter and/or intra molecular bonds were formed. This caused aggregation and insolubilization of actomyosin, this has been showed by Tejada *et al* (1996) in cod minces during frozen storage. Matsumoto (1980) reported that denaturation of actomyosin during frozen storage was a result of aggregation caused by the progressive increase of intermolecular cross-linkage due to formation of hydrogen bonds, ionic bonds, hydro_ phobic bonds and disulfide bonds.

The lowest extractibility of actomyosin found in lot A (100 % minced hake), is related with the high amounts of formaldehyde (FA) formed in this lot (figure 3). It is postulated that FA thus react with the proteins causing denaturation and aggregation (Del Mazo *et al* 1994). Sotelo, *et. al* (1995) stated that the production of FA during frozen storage of gadoids was postulated to be the major factor in protein denaturation in these kind of fish. Sikorski and Kotakowska (1994) showed that a large accu_ mlation of FA in the muscles of frozen fish is generally accompanied by a decrease in extractability of myofibrillar protein. FA causing denaturation of the protein by binding to their side chain groups, it could lead to increased formation of aggregates, buttressed by noncovalent foreces. In addition, Tarrant (1982) postulated that the extracta_ bility of actomyosin from cod muscle which has been frozen rapidly and held below -30°C does not decrease signifi_ cantly, while when muscle is stored between -15°C and -20°C actomyosin is readily insolubilised, where production of FA by the action of TMAO, resulting in a decrease in the extrectability of myofibrillar and the aggregation process is thought to result from the formation of new ionic and

covalent (disulphide) bonds as well as hydrogen bonds and hydrophobic associations.

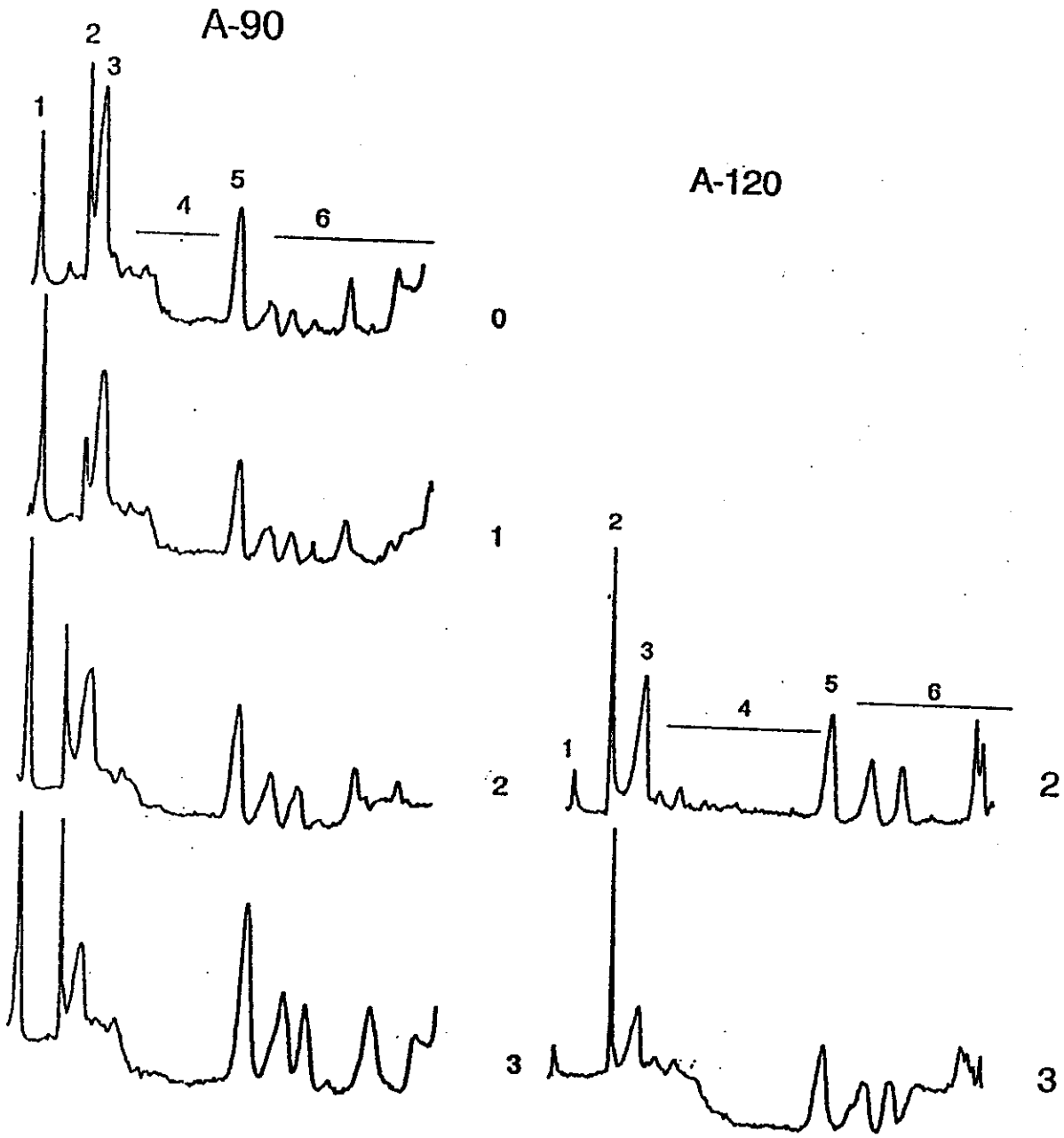
The higher extractability of actomyosin in minced sardine (lot E) when compared with lot (A) seems to reflect the lower concentrations of formaldehyde found in such lot and the higher percentage of lipids in the mince. Mackie (1993) stated that the rate of decrease in extractable protein is dependent upon the species of fish. Shenouda (1980), showed on various fish species an apparent rela_ tionship between their stability and their fat content. Dyer and Dingle (1961) found that lean fish muscles such as lizard, cod, and haddock, with a fat content of less than 1 %, were less stable and showed a fairly rapid decrease in protein (actomyosin) extractability, when compared with fatty fish species (3-10 % lipids) such as yellowtail, halibutt, and rosefish. The protective effect of lipid in fatty fish was associated to the neutral lipid fractions such as triglycerides. Their presence is presumed to diminish or counteract the determintal effect of the free fatty acids. It is assumed that neutral lipid droplets may dissolve the free fatty acids and neutralize their hydro_ phobic effect on protein or compete with them for the binding sites on the proteins as indicated by Shenouda (1980). The protective effects of the lipid was considered to be due to the presumed dissolution of any released free fatty acids in the lipids Mackie (1993), there by neutra_ lizing or diluting their interaction with protein. This mechanism imply that the rate of protein denaturation and aggregation on frozen storage depends on fatty acid pro_ duction and the intervention of neutral lipid. On such a base the proteins of fatty species of fish such as herring and mackerel would have shown the greatest stability.

From the observation of changes after freezing storage of mixtures prepared from hake (lean fish) and sardine, a

correlation between protein extractability, the proportion of minced hake to minced sardine (fatty fish) and the amounts of formaldehyde produced (figure 3) was found. Initially at any rate of hake:sardine the values of extracted of actomyosin were lower than predicted and closer to the one of hake. A protective effect of sardine muscle in the mixtures was observed when sardine ratio increased, but the detrimental effect of hake muscle on extractability was higher than the protective one that expected when adding sardine muscle. As shown in lot B at the lowest amount of minced sardine (25 %) added to minced hake, the greatest amount of formaldehyde were produced (figure 3) and less actomyosin could be extracted with salt solution. So, the protective affect of sardine muscle was counteracted by the highest amount of FA formed in the this lot.

Increasing the ratio of minced sardine to 50 % (lot C) lead to higher quantity of extractable actomyosin compared with lot B at any given time of storage period. Similar results were found as a result of adding higher proportion of minced sardine (75 %) of lot D, where the amount of extractable actomyosin increased when compared with lot C and B during frozen storage at -20°C. These findings proved that presence of lipids may protect the fish proteins or increase their resistance to denaturation during frozen storage.

Comparison among lots showed that the patterns of aggregation in hake and sardine muscle generally differed during frozen storage; no NAM was extracted from lot A by 0.6M NaCL from the fifth month, while NAM extracted from sardine muscle had fallen to half of its value by the end of storage. Such differences in extractability with respect to species having similar characteristics had been reported previously by authors; i.e (Careche and Tejada, 1990 a and 1991, Huidobro and Tejada, 1995). In the mixed hake/sardine



Figure(14) SDS-PAGE of NAM from lot A (100 % minced hake) extracted at (0) zero-time, (1) month, (2) two month, and (3) three month of storage at -20°C. 1. application zone; 2. peak in the stacking/resolving interphase; 3. myosine heavy chain; 4. proteins of MW between 200 and 45 kDa; 5. actin; 6. tropomyosin + troponins + myosin light chains.

more apparent after 3 months of storage at -20°C. Actin (peak 5) is considered to be stable in the extracts during frozen storage until the thrid month, though the total amount of actin extracted was reduced. A similar finding was cited by Tejada *et al*, 1996. The bands corresponding to the tropomyosin, troponins and myosin light chains (peaks of zone 6) increased considerably in the extract after the thrid month of storage at -20°C as shown in the same figure due to the lower amount of MHC and actin that aggregate and are not extracted in 0.6 M NaCl. An increase of bands 1 and 2 soluble protein of high molecular weight that not enter the gel were also observed. These bands were formed by proteins forming bonds as they did not break in the electrophoretic conditions used (SDS + ß ME). When the electrophoresis run was extended at 120 min. a decrease of peak 1 and the corresponding increase of peak 2 was observed. This means that the soluble aggregates retained in the stacking gel were of a molecular weight that enter the stacking gel, but not the resolving gel when electrophoresis run was extended.

In fish species that formed formaldehyde (FA), the disappearance of bands in the resolving gel could be caused by the formation of protein aggregates which would not enter the gel as reported by Huidobro and Tejada (1995). Ohnishi and Rodger (1979) stated that after 12 weeks frozen stored cod at -29°C, there was no MHC band on SDS gel elec_ trophoresis performed on the first peak from gel filtration, though there was a protein band on the gel top. This could be correlted with the protein forming aggregates which were too large to enter the gel during electro_ phoresis. Research carried out by Matthews *et al.* (1980) on frozen stored minced cod and Owusu_Ansah and Hultin (1986) on red hake homogenates showed the decrement of MHC within the extension of storage period. The findings of Laird and Mackie (1981) on cod frames and mince held at -15°C for 18 months showed a disappearance of myosin followed by a

concomitant increase in the low molecular weight proteins. In addition, the amount of actin appeared to remain constant. However, no changes in electrophoretic separation of actomyosin (AM) were observed by Jiang *et al.*(1987) during frozen storage. They also proved that the molecular weight of myosin heavy chain and actin decreased after freezing and going further during frozen storage at -20°C. They suggested that some hydrolysis in MHC and actin of myofibrillar proteins occurred during freezing, frozen storage, and thawing.

The eletrophoretic profiles for lot E (100 % minced sardin) (fig. 15) showed the MHC and actin bands were less intense than in lot A (100 % hake). There was also a marked decrease in TM and TN + MLC when compared with lot A. The MHC band had practically disappeared by the end of storage after 12 months of storage at -20°C appearing as a shoulder of peake 2. Changes in the actin band during frozen storage were less evident. However during frozen storage increase of the soluble protein do not entering the stacking gel (peak 1) and of the protein retained between the stacking and resolving gels (peak 2). This means that the majortiy of the proteins extracted in sardine were forming aggregates of proteins bonds by covalent bonds, beacuse it was not possible to separate in isloated proteins even in the aggressive conditions of electrophortic treatment of the extracts. In the initial extracts a peak in the 4 zone, of MW lower than MHC was observed, this peak do not appeared in the hake muscle. Similar findings were given by Huidobro and Tejada (1995) who stated that in the samples of mackerel (a fatty species), the MHC and A bands weakened during storage at -18°C. The changes in the electrophoretic profile for lot E may be due to protein-lipid interactions causing the formation of covalent bonds among the major proteins extracted.

In lots B (75 % hake + 25 % sardine), C (50 % hake +

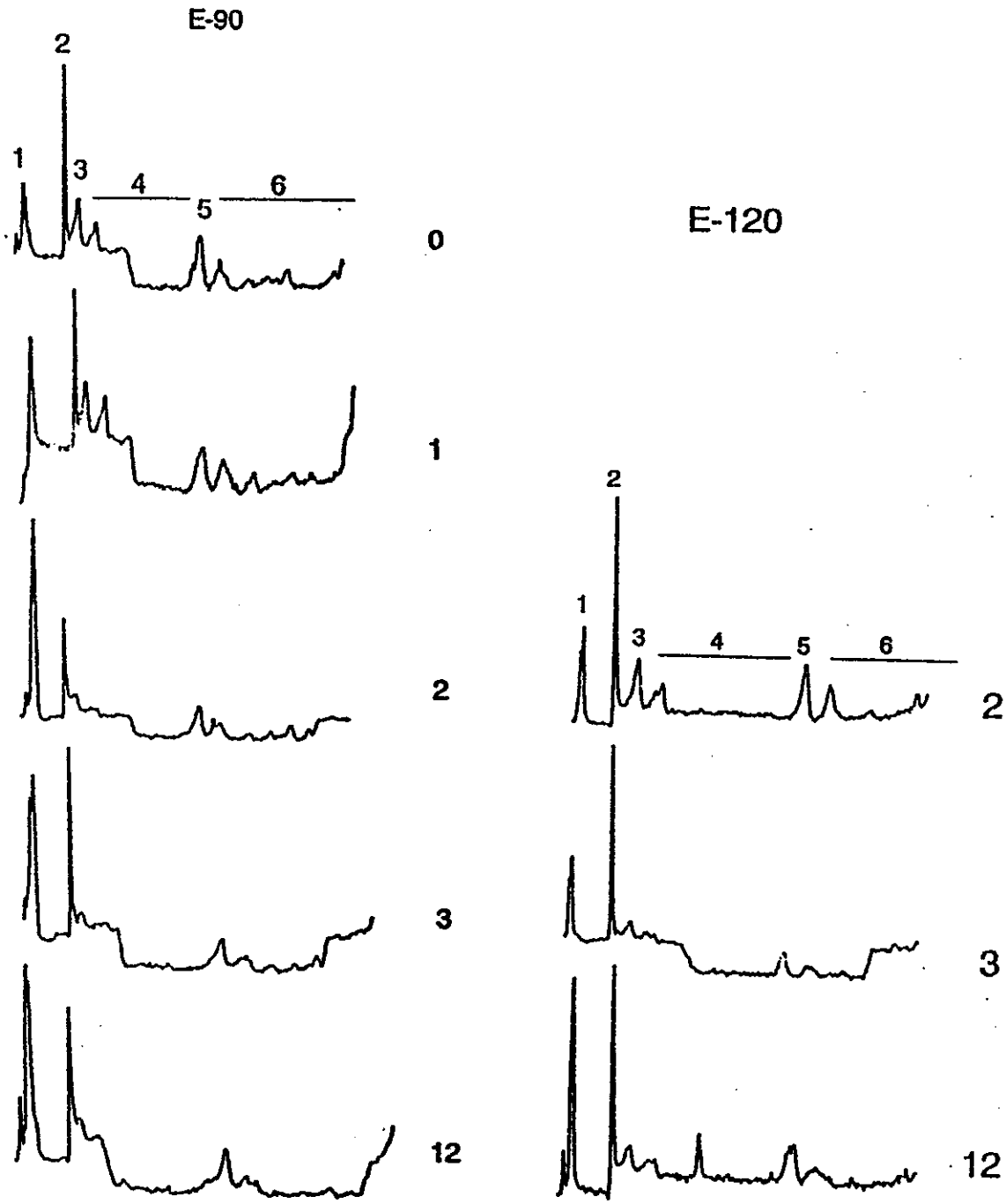


Figure (15) SDS-PAGE of NAM from lot E (100 % minced sardine) extracted at (0) zero-time, (1) month, (2) two month, (3) three month. and (12) end of storage at -20°C. 1. application zone; 2. peak in the stacking/resolving interphase; 3. myosine heavy chain; 4. proteins of MW between 200 and 45 kDa; 5. actin; 6. tropomyosin + troponins + myosin light chains.

50 % sardine) and D (25 % hake + 75 % sardine) figures (16, 17 and 18) the electophorretic profiles of the extracted protein are quite similar. In all of them MHC is evident at zero time (as in the hake) decreasing when storage progressed and appearing as a shoulder of peak 2, similar to the one observed in lot E at the end of the storage period.

The alter in the electrophoretic pattern of the investigated lots differed considerably according to fish species and the ratios of mixture. The results indicated that the electrophortic patterns for the myofibrillar proteins for both species changed during frozen storage. However, changes in myofibrillar proteins are mainly in myosin and the results show that as the times of frozen storage extended the amount of aggregated proteins at the top of the gel increased indicating that very high molecular weight components were accumulated. Same finding was stated by Laird and Mackie (1982), Tejada *et al*.1996 and Torrejón (1996).

Comparison among lots showed that The fractions extracted from hake and sardine with 0.6M NaCl differed from the initial control onward. In hake, the majority peaks were for myosin heavy chain (MHC) and actin (Ac), while in sardine much of the extracted protein was joined by covalent bonds to form soluble aggregates of high molecular weight, so that the relative amount of MHC and Ac in the extract were smaller.

As frozen storage progressed in lot A (100 % hake), soluble aggregates increased, but more significant was a decrease in the MHC band and a relative increase in Ac bands and bands of lower molecular weight than Ac. In lot

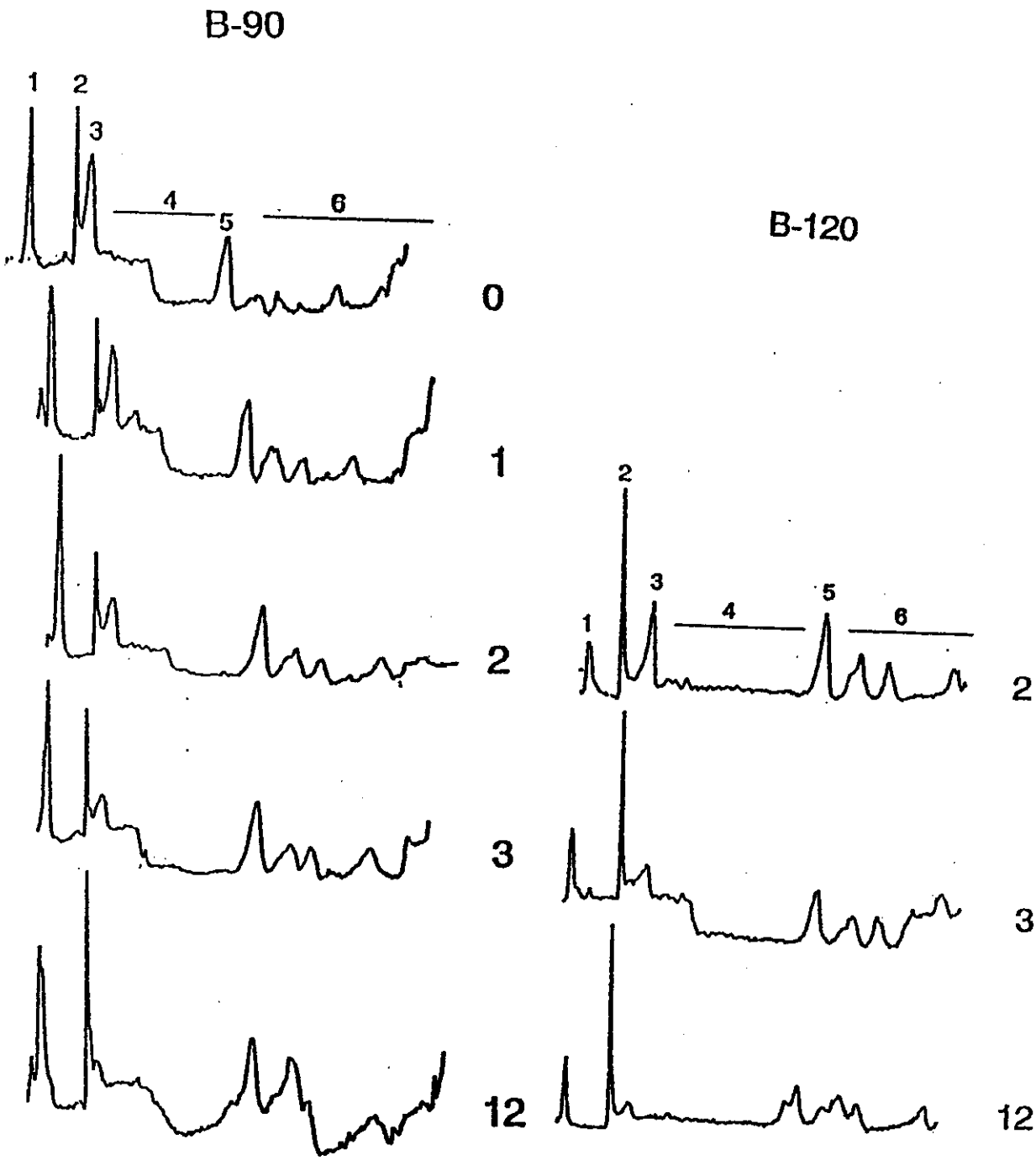


Figure (16) SDS-PAGE of NAM from lot B (75 % minced hake + 25 % minced sardine) extracted at (0) zero-time, (1) month, (2) two month, (3) three month. and (12) end of storage at -20°C. 1. application zone; 2. peak in the stacking/resolving interphase; 3. myosine heavy chain; 4. proteins of MW between 200 and 45 kDa; 5. actin; 6. tropomyosin + troponins + myosin light chains.

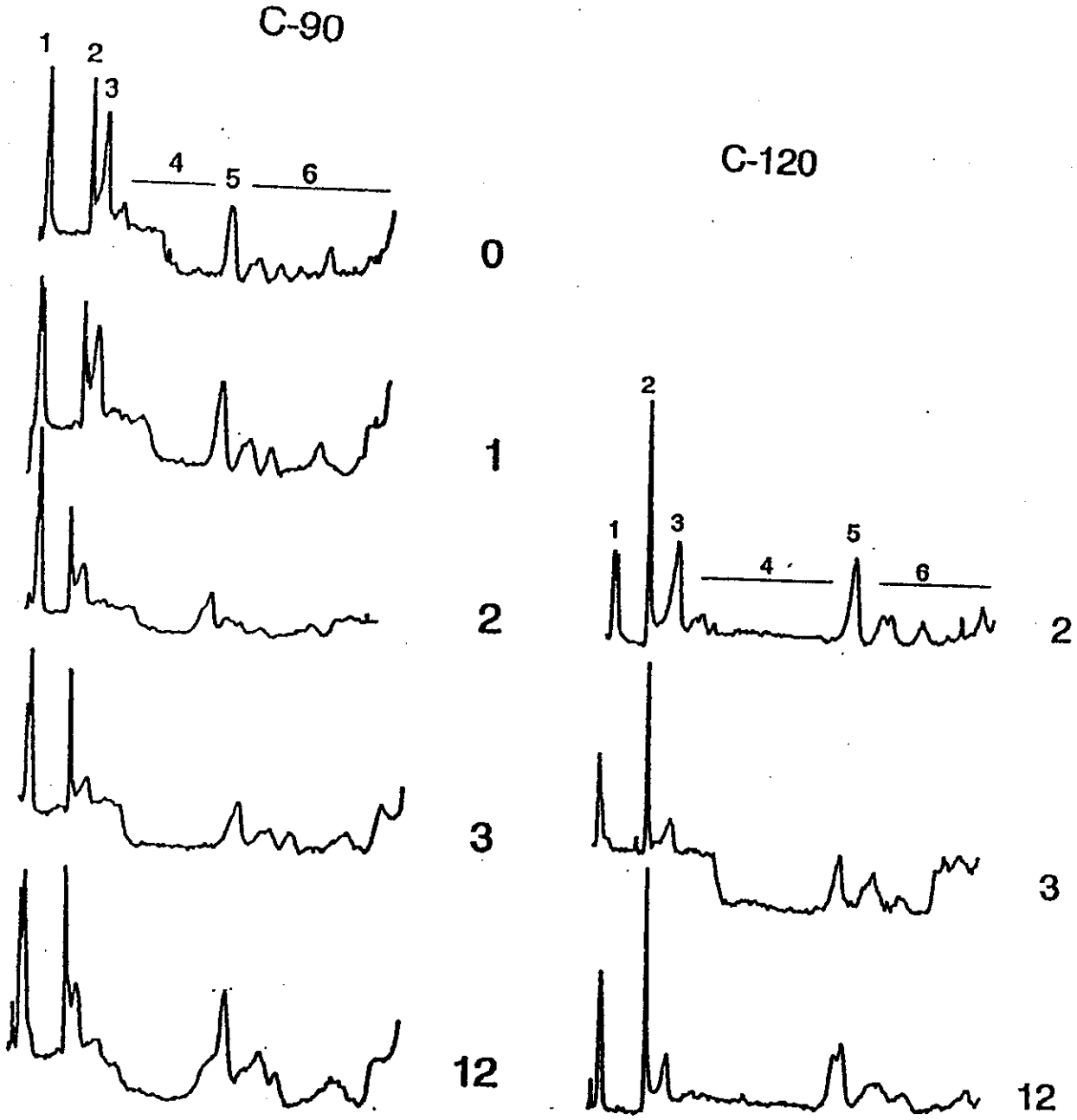


Figure (17) SDS-PAGE of NAM from lot C (50 % minced hake + 50 % minced sardine) extracted at (0) zero-time, (1) month, (2) two month, (3) three month. and (12) end of storage at -20°C. 1. application zone; 2. peak in the stacking/resolving interphase; 3. myosine heavy chain; 4. proteins of MW between 200 and 45 kDa; 5. actin; 6. tropomyosin + troponins + myosin light chains.

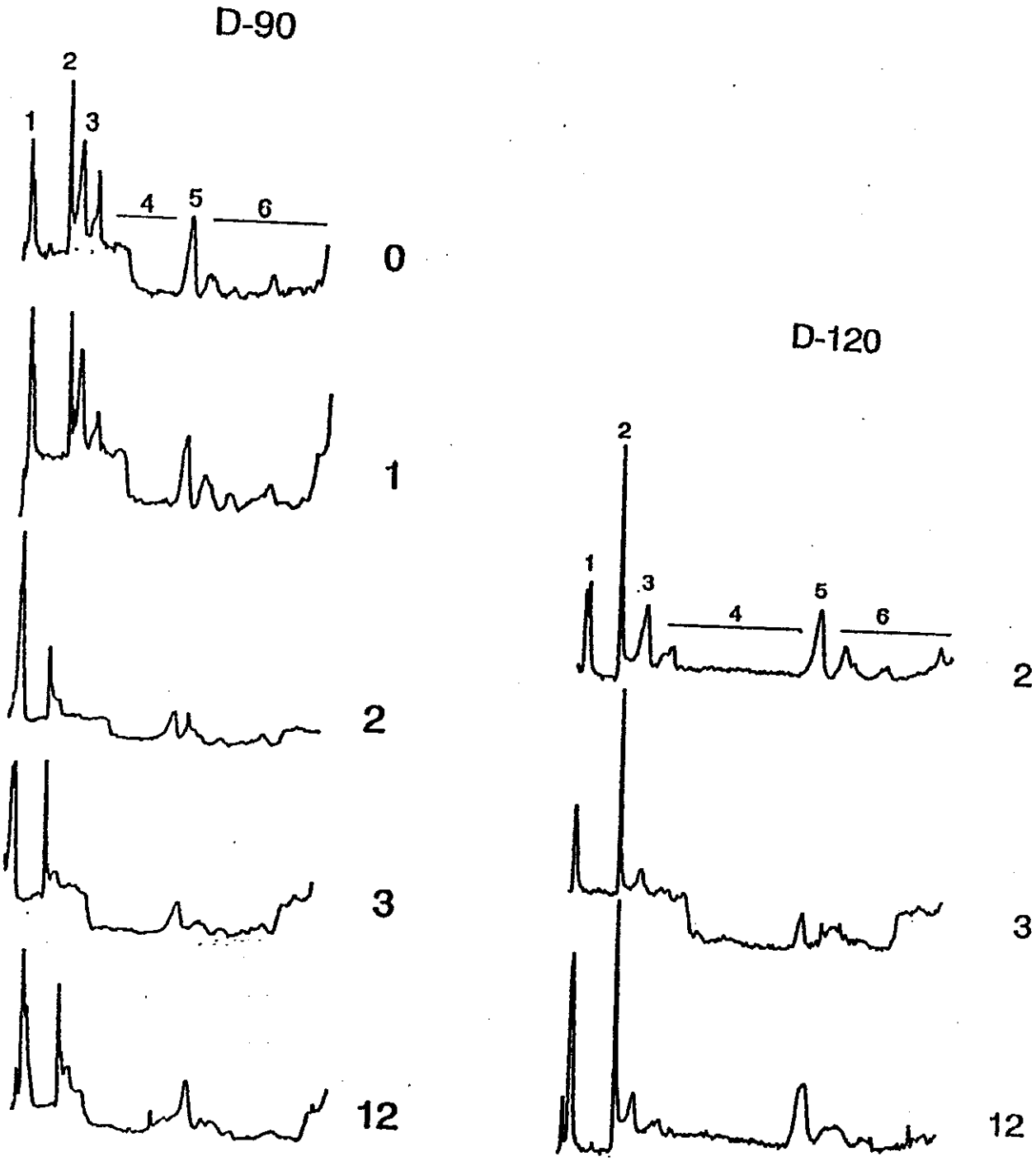


Figure (18) SDS-PAGE of NAM from lot D (25 % minced hake + 75 % minced sardine) extracted at (0) zero-time, (1) month, (2) two month, (3) three month. and (12) end of storage at -20°C. 1. application zone; 2. peak in the stacking/resolving interphase; 3. myosine heavy chain; 4. proteins of MW between 200 and 45 kDa; 5. actin; 6. tropomyosin + troponins + myosin light chains.

E (100 % sardine), peak 1 augmented gradually, indicating an increase in 0.6M NaCl-soluble high molecular weigh micro-aggregates which were retained in the stacking gel because of their size. The differences in electrophoresis of lot E may be due to lipid-protein interactions during frozen storage, which could alter on the level of extractable proteins, or cause a change in the mode of electrophoretic displacement of the protein by altering on their charges, as reported by Huidobro and Tejada (1995). At the outset, the electrophoretic patterns of the protein extracted with 0.6M NaCl in mixed muscle lots revealed the presence of aggregates (peak 1 and 2) and showed majority bands of MHC and Ac. A peak of lower MW than MHC was detected in lot D. This peak was also observed in the sardine lot. At the end of storage, the electrophoretic profile showed a decrease in MHC in the extracts, while the actin peak tended to decline further when the percentage of sardine in the lot was increased.

A.3. Emulsifying properties of natural actomyosin(S1):

The emulsification test involves two stages, firstly the ability of proteins to from an emulsion (emulsifing activity index) and secondly, to stablise it (emulsion stability index). Subsequently, its usefulness as an excellent index of protein functionality is recommended. The emulsifying properties of proteins are affected by the hydrophobicity of proteins.

Emulsion activity index (EAI):

The emulsion activity index test is based on the relationship between turbidity of an emulsion and its interfacial area, which in turn is related to the ability of the protein to adsorb and to stabilize the oil-water

interface (Li-Chan *et al.* 1984). EAI, is expressed in m^2/g of protein, and related to the ability of the protein to adsorb at the interface just when the emulsion is formed. Mahmoud (1994) stated that the emulsifying activity is the area of interface stabilized by unit weight of protein, m^2/g. This index is consequently ruled by the rate of adsorption of the protein at the interface.

Fig. (19) shows the changes which took place in the emulsion activity index. It is evident that in lot A (100 % minced hake), the EAI of extracted actomyosin (5 mg/ml) remained quite constant up to the second month of storage at -20°C, while in the thrid month of storage in which the concenteration of extracted actomyosin was 2 mg/ml, the EAI of lot A showed the highest emulsifying activity when compared with other lots at the same concentration of extracted actomyosin. After fifth month of storage and until the end of storage, no extracted actomyosin was obtained from minced hake, due to this fact no measurement of the EAI was performed in this lot.

In case of lot E, (100 % minced sardine) a good emulsifying activity was exhibited and being slightly superior than other lots. The emulsifying activity at a concentration of 5 mg/ml of actomyosin was maintand up to the second month of storage at -20°C, followed by a decre_ mental trend up to the end of storage. Similar trend was observed at the concentration of 2 mg/ml of isolated actomyosin.

Changes in the emulsifying activity during storage of minced hake at -20°C for 12 months as affected by adding different ratios of minced sardine are given in the same figure. From the obtained data, lot B that contains (75 %

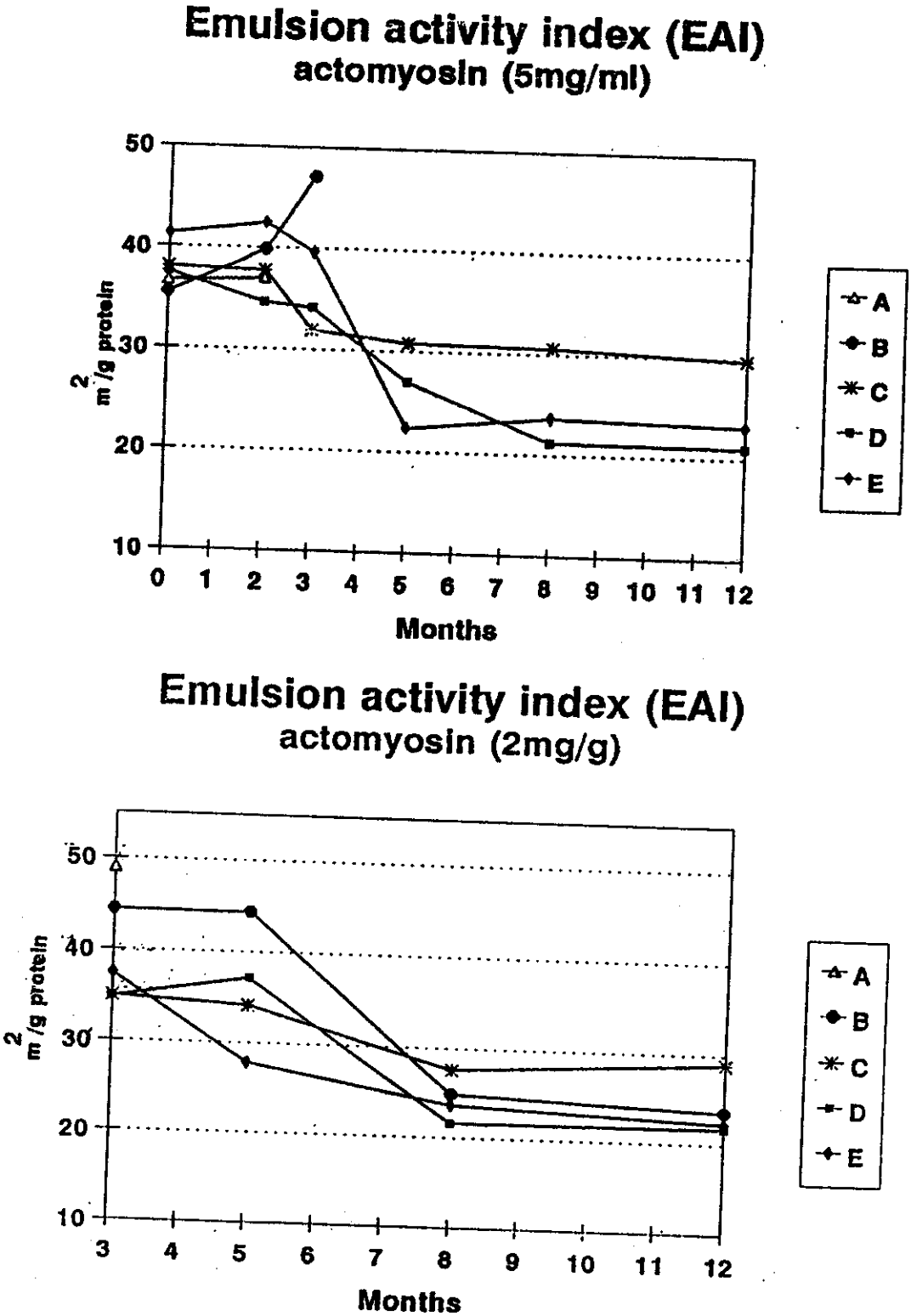


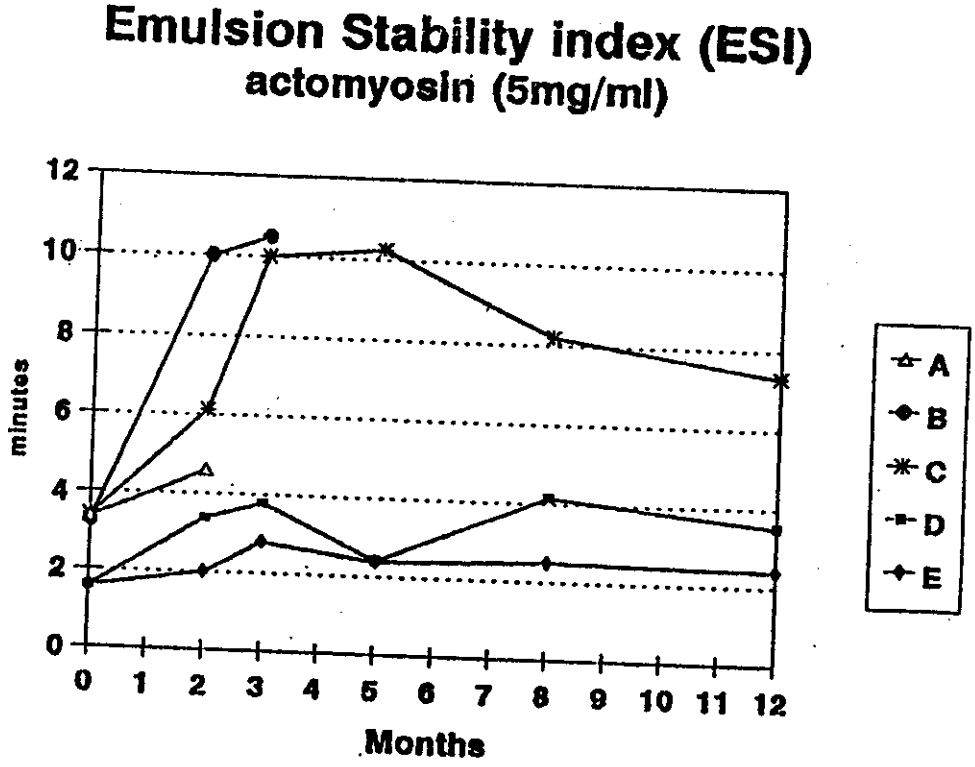
Figure (19). Changes in emulsion activity index (EAI) of actomyosin extracted from different lots during frozen storage at -20°C for 12 months. Lot A: 100 % mince hake; Lot B: 75 % mince hake + 25 % mince sardine; Lot C: 50 % mince hake + 50 % mince sardine; lot D: 25 % mince hake + 75 % mince sardine; Lot E: 100 % mince sardine.

minced hake + 25 % minced sardine) showed higher incre_ mental pattern in the EAI at concentration of 5 mg/ml of actomyosin up to the third month of frozen storage after that, the concentration of the extracted NAM was lower than 5 mg/ml. A sharp decrease of EAI at the concentration of 2 mg/ml of actomyosin was observed from fifth month up to the end of the storage period (fig.19). The emulsion activity index of lot C (50 % minced hake + 50 % minced sardine) exhibited no evidence of changes in EAI up to the second month of storage and then showed a downward trend maintaing the values stable until the end of storage period. The same trend was found when the concentration of actomyosin was 2 mg/ml. The EAI of lot C was the highest obtained from 5th month up to the end of the storage period a fact that was observed were clearly at a concentration of protein of 5 mg/ml. The emulsifying activity of lot D (25 % minced hake + 75 % minced sardine) decreased gradually during frozen storage obtaining EAI similar to that of lot E at the end of storage period.

Emulsion stability index (ESI):

The flexibility of proteins, is very important to explain their ability to form the emulsion by spreading over the interfacial layer. Stability of the emulsion are more related to the rheological properties of the layer and to the electrostatic repulsions between the droplets.

Figure (20) show the emulsion stability of extractable actomyosin of different lots during frozen storage. The obtained results clearly showed that the creaming stability of lots A, B and C are initially larger than those of lots D and E. The data indicated that the emulsion stability of lot A increased from 3'36" min. at zero time to 4'6" at the second month of storage at -20°C at a concentration 5 mg/ml of actomyosin, maintaing similar values at the third month



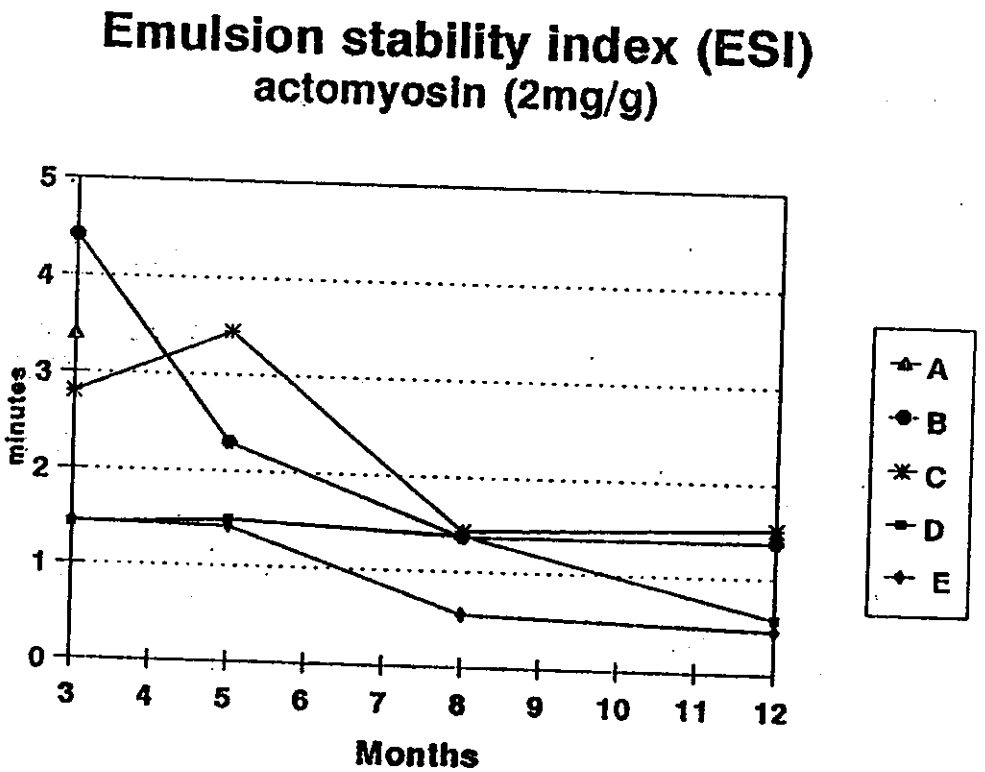


Figure (20). Changes in emulsion stability index (ESI) of actomyosin extracted from different lots during frozen storage at -20°C for 12 months. Lot A: 100 % mince hake; Lot B: 75 % mince hake + 25 % mince sardine; Lot C: 50 % mince hake + 50 % mince sardine; lot D: 25 % mince hake + 75 % mince sardine; Lot E: 100 % mince sardine.

of storage at -20°C when the emulsion stability of actomyosin was measured at a concentration of 2 mg/ml.

The stability of the emulsion of lot E (100 % minced sardine) toward coalescence exhibited very poor emulsion stability since during frozen storage it showed nearly a steady state of emulsion stability of extractable acto_ myosin (5 mg/ml) until the end of storage period except at the third month that a increase was observed, a decrease of ESI was obtained from 8th month with a concentration of protein at 2 mg/ml.

In case of lot B (75 % minced hake + 25 % minced sardine), the stability of emulsion of extractable acto_ myosin (5 mg/ml) was 3'24" min., and after the second month of storage the emulsion stability reached to 10 min., after which increased slightly at the third month of storage. When the ESI was measured at 2 mg/ml protein concentratian, a decrease was observed up to 8th, remaining stable until the 12th month. Lot C (50 % minced hake + 50 % minced sardine) exhibited a stabilization index that increased gradually from 3'34" to 10'24" and after fifth month of storage at -20°C decreased slowly up to the end of storage. Lot C maintains in general the highest and more stable values of EST at 5 mg/ml and the highest at 2 mg/ml at 5th month. As the ESI depends on protein concentration and ability of the protein to retain the oil, it means that the natural actomyosin extracted in this lot maintains the best characteristics of all the lots concerning the ability of the extracted protein to emulsify and stabilize the emulsion. On the other hand, extracted actomyosin (5 mg/ml) of lot D (25 % minced hake + 75 % minced sardine), the emulsion showed a lower stabilization effect when compared with lots C and B. The values of ESI of lot D at 2mg/ml were similar to the once obtained in lot E, except at 8 month of storage at -20°C.

affects protein functionality. However, the available data proved that emulsifying properties of these samples can be improved by denaturation. Morr (1979) reported that improvement in functionality is probably due to an unfolding of the molecule to expose hydrophobic amino acid residues, thus making the protein more amphiphlic and capable of orienting at the oil-water interface. So the higher EAI value is probably due to the increment of their hydrophobicity. Damodaran (1994) stated that partial denaturation of proteins that does not cause insolubi_ lization improves emulsifying properties of proteins. This is attributable to an increase in the surface hydropho_ bicity. However, excessive denaturation that cause a decrease in solubility often results in poor emulsifying properties where lot B has less protein in the extract and also has a lower ESI.

Both lots D and E were very similar which showed lower emulsifying properties during the end of frozen storage indicated that the membrans formed by salt soluble proteins were brittle as the result of altering these proteins where changing the type of protein involved and the myosin which composing the interfacial protein (IPF) was not enough and fat separation occurred. Borejdo (1983) determined the hydrophilic and hydrophobic sites on a myosin molcules, showing that the heavy meromyosin (HMM) has high hydrophobic areas, and he suggested that the HMM portion faces the fat globules, whereas light meromyosin (LMM) faces the water phase. Smith (1988) suggested that protein aqueous membrane deposits surrounding a fat globule stabilize and myosin appears to be the major protein involved in protein coating of fat droplets.

B.1. **Extractability of aggregates (P1 Fractions):-**

The myofibrillar proteins of many fish species aggregate during frozen storage (Matsumoto and Noguchi, 1992), with considerable alteration on some functional properties and the texture of the muscle. When this occurs in many species of high commercial value, it spells the end of practical storage life. These studies underline the importance of secondary interactions and disulfide bridges, which are considered the chief cause of myofibrillar protein aggregation. For this purpose, the changes occurring in insoluble resdue obtained from extraction natural actomyosin (NAM) with different agents capable of selectively breaking different protein bonds were examined periodically during frozen storage at -20°C. It have been investigated to elucidate the mechanisms of protein aggregation during frozen storage, it was hoped to ascertain which bonds are responsible for the formation of aggregates and which are the major proteins extracted with each of the agents.

The solubilization of aggregated proteins was conducted by using various agents which differ from each other according to their ability to cleave intermolecular bonds; namely electrostatic (SDS), hydrogen and hydrophobic (urea), and S-S bonds (ß-mercaptoethnol). Haard (1992 a) showed that loss of protein extractability during frozen storage of fish can mostly be accounted for by non-covalent, hydrophobic interaction. Much of fish protein which was denatured during frozen storage; is soluble in sodium dodecyl sulphate (SDS) and more than 95 % is soluble when a disulphide bond reducing agent like ß-mercaptoethanol is combined with SDS in the solvet.

B.1.a. **Breaking down secondary interactions:**

* **Treatment with 2 % SDS:**

Treatment of protein unextracted by 0.6M NaCl (precipitates P1) with 10 % SDS for 20 hours produced no significant increase in the percentage of solubilized protein versus protein extracted with 2 % SDS and 10 min stirring (results not shown). The latter treatment was therefore considered adequate to breakdown secondary bonds (hydrogen bonds and hydrophobic interactions) in the aggregates.

After five months' storage at -20 °C the amount of protein extracted by 0.6M NaCl differed according to species and mixture (Fig. 13); however, upon breakdown of bonds with 2 % SDS the amount of protein extracted from the P1 fractions was 26.7 % in 100 % hake (Lot A) as compared to 44.4 % in 100 % sardine (lot E) (Fig. 21). In the mixed lots (B, C and D) the percentage extracted from the aggregate was similar to that extracted from the sardine lot (Lot E).

In all lots the amount of NAM extracted from aggregate P1 upon breakdown of secondary interactions with 2 % SDS declined as storage progressed. By the end of storage, practically no protein was extracted from the aggregate.

* **Treatment with 2 % SDS + 8M Urea:**

When the P1 aggregates from the different lots were treated with 2 % SDS + 8M urea, the amount of NAM extracted from precipitate P1 declined as storage progressed (Fig. 21). But although both SDS and urea breakdown secondary interactions, the amount of protein extracted with these agents and with 2 % SDS differed: in lots A and C at 5 months,

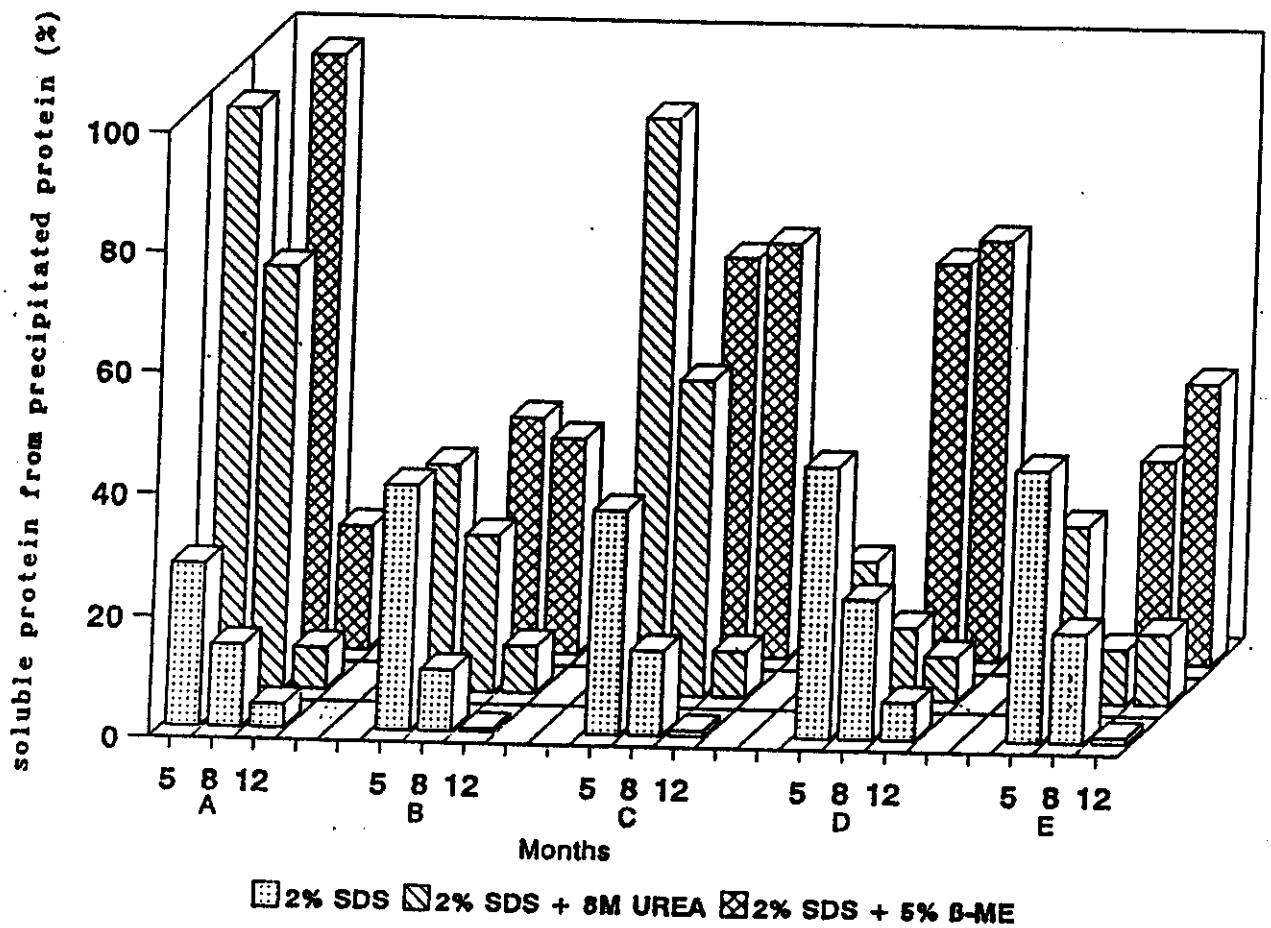


Figure (21). Protein extracted (S1) in 2 % SDS, 2 % SDS + 8M Urea, 2 % SDS + 5 % ß-mercaptoethanol (ME) from precipitated protein insoluble in 0.6M (P1) NaCl from different lots at 5, 8 and 12 months of frozen storage at -20°C.

protein extraction values were 95.6 % and 95.2 % of protein in P1, respectively as compared to 26.7 % and 36.7 % with 2 % SDS alone. In lots B, D and E, on the other hand, the respective percentages were 36,97 %, 22,54 % and 28,84 % (Fig 21), which in the last two cases were less than the extraction percentage with 2 % SDS. Although both 2 % SDS (Tejada *et al*, 1996) and 8M urea (Tsuchiya *et al*, 1979) are known to breakdown secondary interactions, the reason why more protein was extracted from the aggregates in lots A and C with 2 % SDS + 8M urea than with 2 % SDS alone may be attributed to that the structure of the aggregates forming in these lots was different from that of the aggregates forming in lots B, D and E. Two types of action have been described for urea when it is used to solubilize proteins: on the one hand, the advantage of urea for some applications is that it does not affect the intrinsic charge of proteins and so separation of the constituent polypeptides will be on the bases of both size and charge, in contrast to the use of SDS (Hames, 1994). In fact, because of its lower MW, urea can reach locations in the aggregates which SDS cannot, and thus more protein (fraction S2) was extracted from aggregate A1. However, other authors have reported urea-induced SH-SS interchange reactions in proteins (Xiong and Kinsella, 1990), which ought likewise to augment extraction. These two types of action, occurring according to the type of aggregate, may account for the difference in the amounts of protein extracted with 2 % SDS and 2 % SDS + 8M urea.

After 8 months' frozen storage, the amount of NAM extracted from aggregate P1 formed in the minced lots was greater than was extracted with 2 % SDS in lots A, B and C and smaller than with 2 % SDS in lots D and E.

At the end of storage, more protein was extracted with 2 % SDS + 8M urea than with 2 % SDS alone in all cases. The largest amount was extracted from lot E (11.49 %) and the smallest from lot A (6.79 %). There was very little difference in the amounts of protein extracted from aggregates in lots with both type of muscle (B, C and D).

Similar amounts of NAM were extracted with 2 % SDS + 8M urea from the aggregates in lots C and A (100 % hake), which would indicate that more of the protein in lots A and C was bound by secondary interactions up to the 8th month of storage, whereas the pattern in lots B and D was more akin to that of lot E (100 % sardine), with greater initial involvement of covalent bonds.

B.1.b. Breaking down secondary interactions and disulfide bonds:

*** Treatment with 2 % SDS + 5 % β-ME:**

1) Lot A: When the aggregate P1 was treated with 2 % SDS + 5 % β-ME (Fig 21), at 8 months' storage, although all the NAM was aggregated (Fig 13), 98 % of the protein in fraction P1 from lot A was solubilized. This figure had dropped to 20 % by the end of 12 months' storage. A smaller percentage of protein than in the other lots was extracted from the lot A aggregate by the end of storage; this indicates the importance of the initial incidence of secondary interactions and disulfide bonds in the hake aggregates and agrees with the results of treatment with urea. By the end of storage other covalent bonds had formed which prevented extraction of

the aggregated protein. Similar results have been reported in minced cod by Tejada *et al* (1996) and in hake by Torrejón (1996).

2) Lot E: In this lot the amount of protein extracted from the aggregate with 2 % SDS + 5 % β-mercaptoethanol at 8 months was much smaller in sardine than in lot A; the given extraction conditions were insufficient to allow full release, and 67 % of the protein remained unextracted. This suggests that covalent bonding occurred earlier in the sardine than in the hake aggregate, even although the actual amount of the aggregate was much smaller in sardine (Fig 13).

3) In the lots with mixtures of the two species, the amount of protein extracted by breakdown of secondary and disulfide bonds remained practically unchanged at around 67 % in lots C and D and 38 % in lot B (Fig 21). In all lots breakdown of secondary and disulfide bonds with this treatment increased the amount of protein extracted from aggregate P1 as compared to the other solubilization treatments, at both 8 and 12 months' storage. This was most evident in lot D, followed by lots C and B. The amount of extracted protein upon breakdown of secondary and disulfide bonds remained stable until the end of storage in all lots containing sardine. This was an indicative of the importance of non-disulfide covalent bonds in the aggregate when sardine muscle was present.

The behaviour of lots B (hake:sardine= 3:1) and C (hake:sardine= 1:1) was unexpected: although protein aggregation in lot B was close to 100 % (comparable to the hake lot) and intermediate in lot C (Fig 13), with respect to the percentage of protein extracted from aggregate P1

(insoluble in 0.6M NaCl) (Fig 21) the type of aggregate which formed in lot B behaved like that of the sardine lot when disulfide bridges were broken down with 2 % SDS + 5 % ME. The percentage of protein extraction was lowest in these two lots. This would indicate that the percentage of non disulfide covalent bonding of NAM in P1 aggregates was higher in lot B than in the other mixed muscle lots.

In lot C there was high incidence of protein aggregation through secondary and disulfide bonding, whereas in lot D, although the amount of protein extracted with 2 % SDS and with 2 % SDS + 5 % ME was similar to the amount extracted from lot C, there was a considerable difference in the amount extracted with urea. This suggests that the types of aggregate that formed were different.

B.2. **Polyacrylamide Gel Electrophoresis (SDS-PAGE):**

* **Protein extracted with 2 % SDS:**

The electrophoretic profiles of the S2 fractions (extracted with 2 % SDS from aggregates P1 insoluble in 0.6M NaCl) are shown in Fig (22).

Lot A: In this lot, at 5 months' storage the protein extracted upon breakdown of secondary interactions consisted mainly of myosin heavy chain (MHC) (peak 3) and actin (Ac) (peak 5). As storage progressed the amount of MHC extraction declined, and by the end of 12 months the Ac peak was the largest. There was an increase in the band retained between

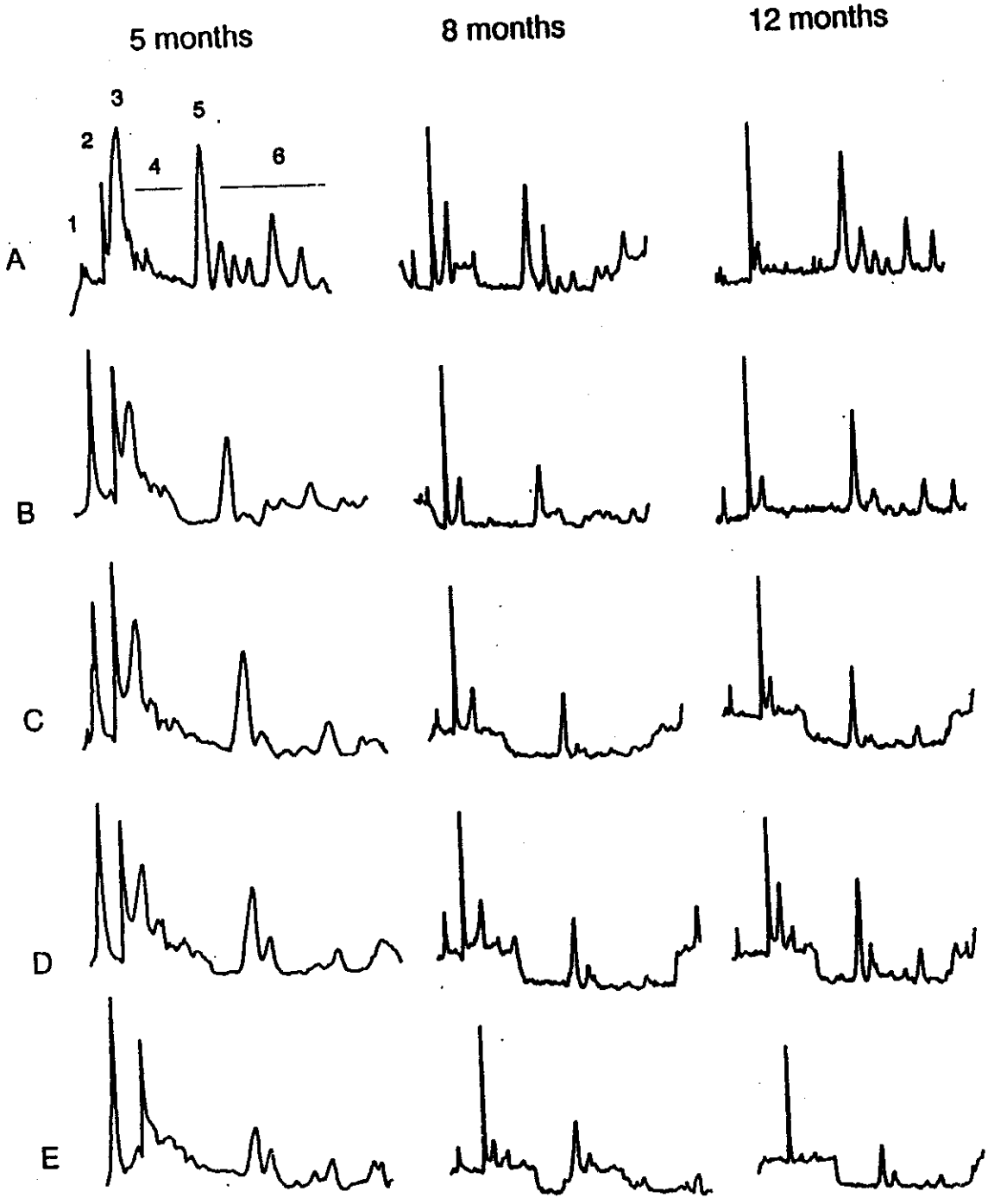


Figure (22) SDS-PAGE of different lots of fractions S2/p1 from different lots extracted at 5, 8, 12 months of storage at -20°C and treated with 2 % SDS. (1) application zone; (2) peak in the stacking/resolving interphase; (3) myosine heavy chain; (4) proteins of MW between 200 and 45 kDa; (5) actin; (6) tropomyosin + troponins + myosin light chains.

the stacking and the resolving gel (peak 2), reflecting the extraction of high-MW soluble aggregates.

Lot E: In this lot the protein extracted at 5 months from the 0.6m NaCl-insoluble aggregate through breakdown of bonds by 2 % SDS was composed largely of aggregates which did not enter the stacking gel (peak 1) or the resolving gel (peak 2). MHC appeared as a shoulder of peak 2 and there was also an actin peak, although relatively smaller than in other lots. This suggests that in lot E when secondary interactions were broken down by 2 % SDS, the micro-aggregates in the extracted protein were linked by covalent bonds, given that these were not broken in the preparation of the sample for electrophoresis. At 12 months' storage the majority band consisted of aggregates of intermediate molecular weight, which did not enter the resolving gel (peak 2) but were not retained in the stacking gel.

Values for mixed-species lots (B, C and D) were intermediate between lots A and E. At the outset there were protein aggregates which did not enter the gel (peaks 1 and 2), and majority bands of MHC and Ac. By the end of storage the majority band was actin, although a MHC peak was visible in all cases.

* **Protein extracted with 2% SDS + 8M urea:**

Fig. 23 shows the electrophoretic profiles of S2 fractions extracted from aggregates P1 (insoluble in 0.6M NaCl) with 2 % SDS + 8M urea.

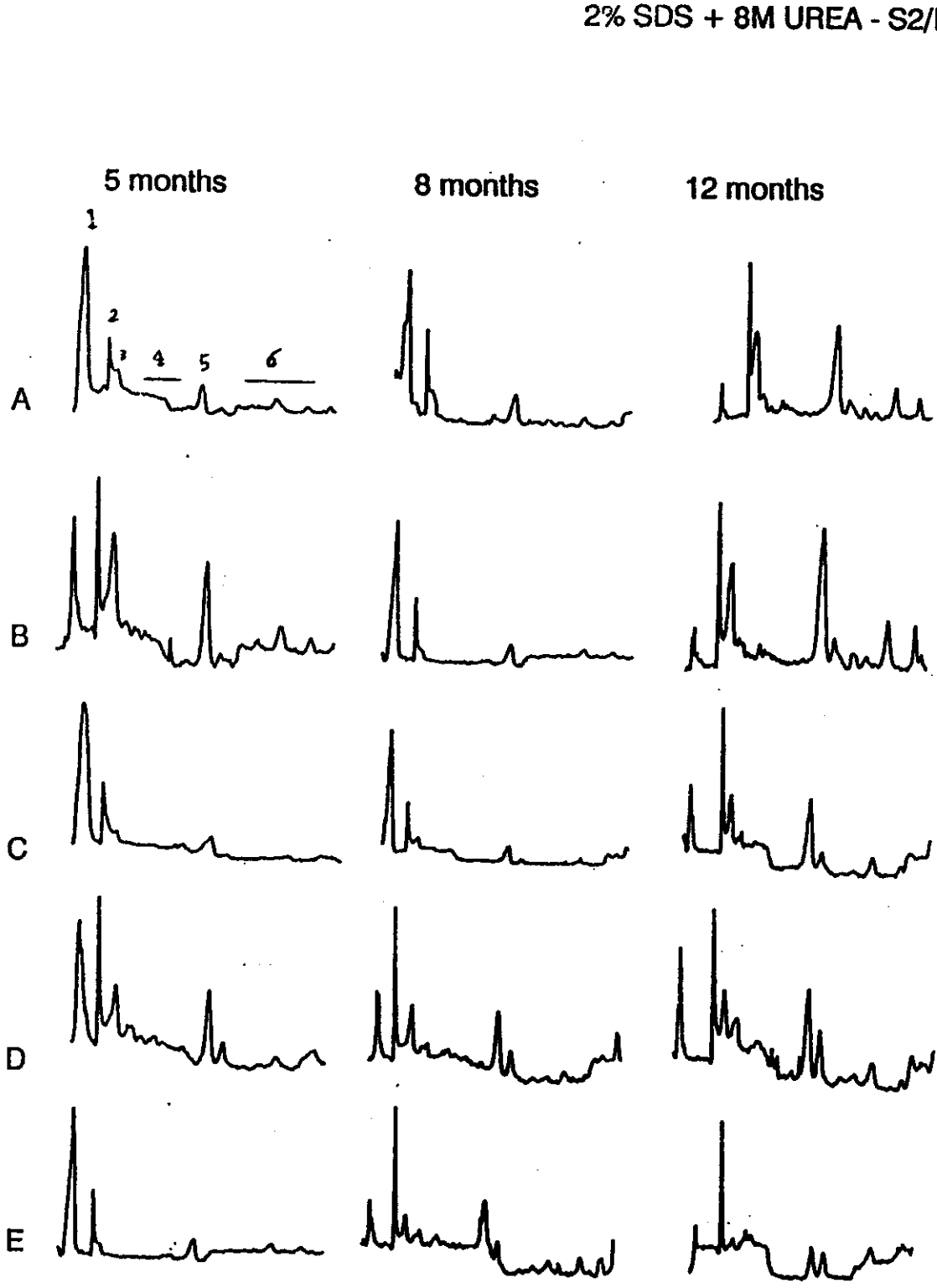


Figure (23) SDS-PAGE of different lots of fractions S2/p1 from different lots extracted at 5, 8, 12 months of storage at -20°C and treated with 2 % SDS + 8M UREA. (1) application zone; (2) peak in the stacking/resolving interphase; (3) myosine heavy chain; (4) proteins of MW between 200 and 45 kDa; (5) actin; (6) tropomyosin + troponins + myosin light chains.

At 5 months a majority high-MW band (peak 1) which did not enter the gel was detected in lots A and C; in lots B and D the profile resembled that recorded with 2 % SDS, with the MHC and Ac peaks clearly visible in the protein entering the resolving gel. This indicates that protein extraction with 2 % SDS and with 2 % SDS + 8M urea was different in lots A and C: the extracted protein with 2 % SDS + 8M urea formed soluble high-MW micro-aggregates partially linked by covalent bonds. The extraction of these microagregates is consistent with the higher level of protein extraction from aggregate P1 with 2 % SDS + 8M at 5 and 8 months (Fig 21). The electrophoretic profiles were similar to those recorded for lot E.

As storage progressed, peak 1 declined in all lots. Suggesting that this treatment extracted less protein because of the formation of other covalent bond and consequently the formation of aggregates of higher MW which precipitated in the experimental conditions (unextracted fractions P2). In this case the electrophoretic profiles were like those obtained from extraction with 2 % SDS alone (Fig 22).

The electrophoretic profile in lots B and D were similar to the profile for 2 % SDS. There was practically no variation after 12 months' storage. The electrophoretic profiles for lot E were similar to the profiles for extraction with 2% SDS only, indicating that there was covalent bonding in the extracted protein. The reason for the higher apparent concentration of Ac and MHC peaks in the protein extracted at the end of storage was that 1μL was applied to the gel at a concentration of 1 mg/mL, which meant that when peak 1 declined there was a change in the relative percentage and the extracted protein became enriched with these proteins.

* **Protein extracted with 2% SDS + 5% β-ME:**

Fig 24 shows the electrophoretic profile at 8 and 12 months where aggregates P1 (unextracted in 0.6M NaCl) were treated with 2 % SDS + 5 % ME. The protein extracted in this fraction was bound in aggregate P1 by secondary and disulfide bonds.

In lot A, although the amount of protein extracted upon breakdown of secondary and disulfide bonds fell sharply between 8 and 12 months (Fig 21), there was practically no change in the electrophoretic profile, where the majority protein formed micro-aggregates linked by covalent bonds (peak 1). The MHC appeared joined to peak 2, while the actin peak was clearly visible at both 8 and 12 months. This electrophoretic pattern was very similar to ones derived from protein extracted with 2% SDS + 8M urea, despite the fact that less protein was extracted with the latter treatment (fig 21)

The behaviour pattern in lot E was similar, although the percentage of peak 1 upon breakdown of disulfide bonds was higher at 8 months. This would indicate that the protein extracted from the aggregates in the sardine lot was obtained largely through the formation of soluble micro-aggregates held together by covalent bonds.

No clear trend was apparent in the electrophoretic profiles of the fractions extracted upon breakdown of secondary interactions and disulfide bonds in the aggregates from lots containing a mix of the two species. The differences between the two extraction treatments were greatest in lot B: at 8 and 12 months' storage the electro_

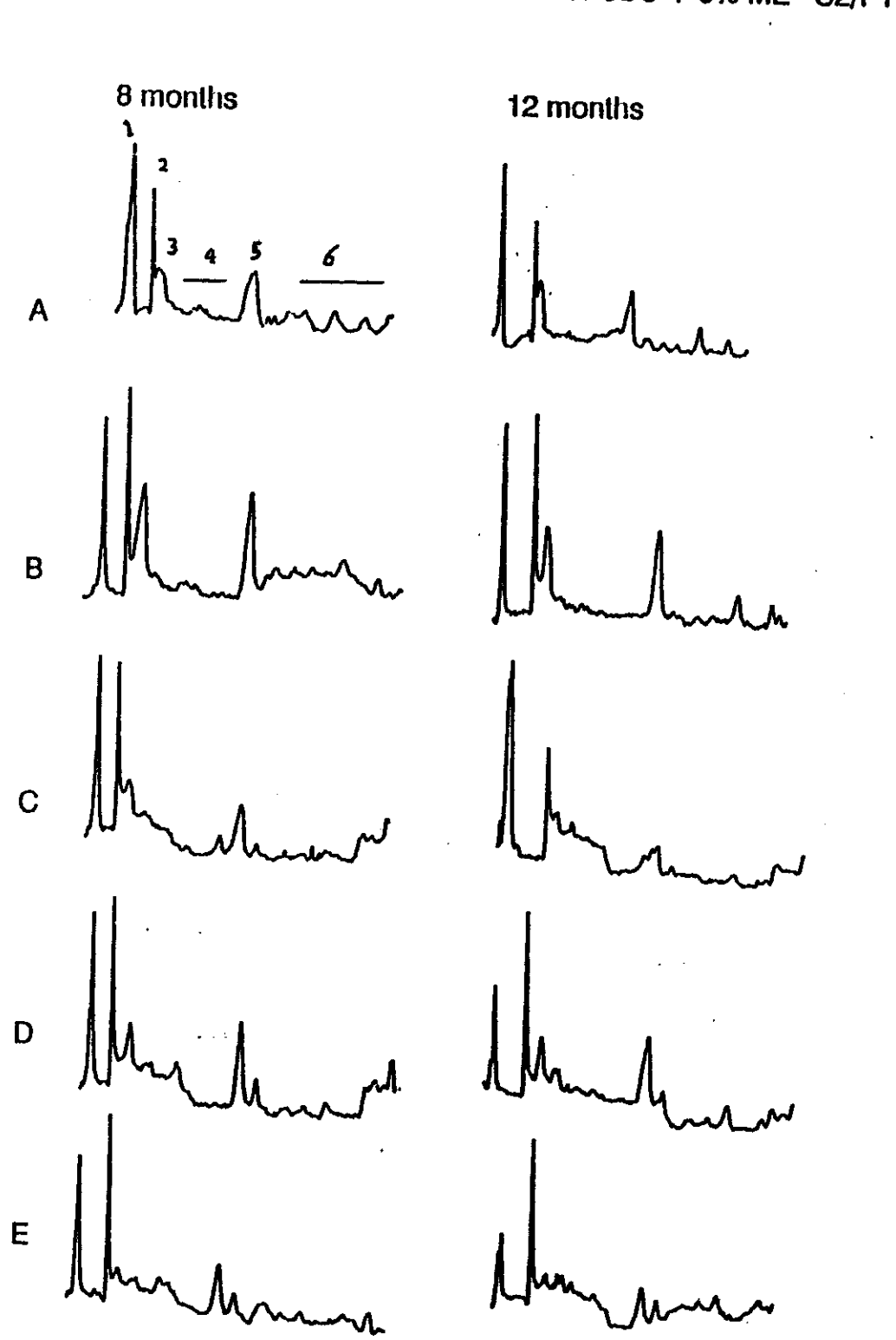


Figure (24) SDS-PAGE of different lots of fractions S2/p1 from different lots extracted at 5, 8, 12 months of storage at -20°C and treated with 2 % SDS + 5 % ME. (1) application zone; (2) peak in the stacking/resolving interphase; (3) myosine heavy chain; (4) proteins of MW between 200 and 45 kDa; (5) actin; (6) tropomyosin + troponins + myosin light chains.

phoretic profiles of the extracts obtained by treating the aggregate with 2 % SDS + 5 % β-ME showed clearly separated MHC and Ac peaks, something which was not observed in the lots extracted with 2 % SDS + 8M urea. This suggests that of the additional amount of protein obtained upon breakdown of disulfide bonds, part was composed of MHC and Ac linked by bonds which brokedown in the experimental conditions.

Type of aggregate

A different type of aggregate formed in each lot, irrespective of the amount of unextracted protein (Fig 13). In hake (lot A) the bulk of the aggregate was extracted with 2 % SDS + 8M urea up to 5 months' storage, and with 2 % SDS + 5 % ME up to 8 months. This was indicative of the importance of secondary and S-S bonds in the formation of aggregates which are unextractable in 0.6M NaCl. However, after 12 months' storage, covalent bonding was detected which prevented extraction of 80 % of the aggregate in the experi_ mental conditions. Flair (1996) reported that the aggregated form of protein in frozen cod, haddock and hake that found in the early period of frozen storage at -20°C were linked by hydrophobic bonds, hydrogen bonds and electrostatic linkages. After 8 months of storage of cod at -20°C and 12 months at -30°C, (and earlier in hake), covalently linked (disulphide bonds) ring-shaped aggregates were formed, and so product texture was affacted.

In sardine muscle (lot E), although the amount of protein extracted with 2 % SDS from the NaCl-unextractable aggregate was smaller than in lot A (Fig 13), the percentage of protein extracted from the aggregate P1 upon breakdown of disulfide bonds was much smaller at 8 months than in the hake lot. This indicates earlier formation in sardine of non disulfide covalent bonds sufficient to prevent extraction of

approximately 60 % of the protein in the aggregate insoluble in 0.6M NaCl.

The difference in extractability of the aggregates forming in either species reflect the different types of aggregation associated with the formation of FA in the muscle and with the presence of lipids. In the case of hake, FA formation in the muscle is related to the early formation of NaCl-unextractable aggregates in which the protein is essentially linked by secondary interactions and disulfide bonds. In sardine, the presence of lipids would induce slow formation of aggregates in which a high percentage of the protein was linked by non disulfide covalent bonds.

In the mixed-species lots, the amount of protein held together by non disulfide covalent bonds was similar at 8 and 12 months' storage, although this percentage was much higher in lot B (75 % hake + 25 % sardine) than in lots C or D and similar to that found in sardine. Lot B exhibited rapid loss of extracta_ bility in 0.6M NaCl, similar to that detected in hake, and there was more FA formation during frozen storage. This could have caused initial unfolding of the protein and rapid denaturation (Del Mazo *et al*, 1994, Torrejón, 1996, Del Mazo, 1997), leaving the protein susceptible to formation of covalent bonds since there were lipids in the medium.

Part IV: Transmission electron microscopy (TEM):

* Muscle morphology:

Figures (25 to 28) show the TEM structure of muscle of hake (Figs 25, 26) and sardine (27, 28) at 2 (Figs 25, 27) and 12 (Figs 26, 28) months of storage at -20°C. We can see how initially the muscle structure was intact in both species, with Z-line clearly visible. As storage progressed, Z-line in the hake became increasingly disorganized, whereas in the sardine there was scarcely any change.

* Supernatant morphology (S1 fractions):

Lot A: Fig 29 shows the natural actomyosin (NAM) extracted in the supernatants (S1 fractions) obtained from hake minces with 0.6M NaCl by the method of Kawashima *et al* (1973) described in materials and methods after 2 months' frozen storage. The protein in the supernatant was in globular form, displaying numerous short filaments in association with mutually-interconnected globules. Also visible were numerous micro-aggregates with which these formed associations. Some filaments were found crossed at angles ranging from 94° to 108°, which suggests the presence of covalent bonds as described by Tejada *et al.*(1996). No natural actomyosin was extracted in this lot at 5 months storage.

Lot B: Figs 30 to 32 show S1 fractions from B minces (75 % hake + 25 % sardine) at 2, 5 and 12 months' frozen storage. At 2 months (Fig 30) the protein in the supernatant exhibited filamentous zones linked by aggregates which were larger than in the previous case. As storage progressed (Fig 31, 32) ring-shaped structures and micro-aggregates appeared which

Fig. 25

hake muscle (x=23000)

2 Months (Bar=0.6 µm)

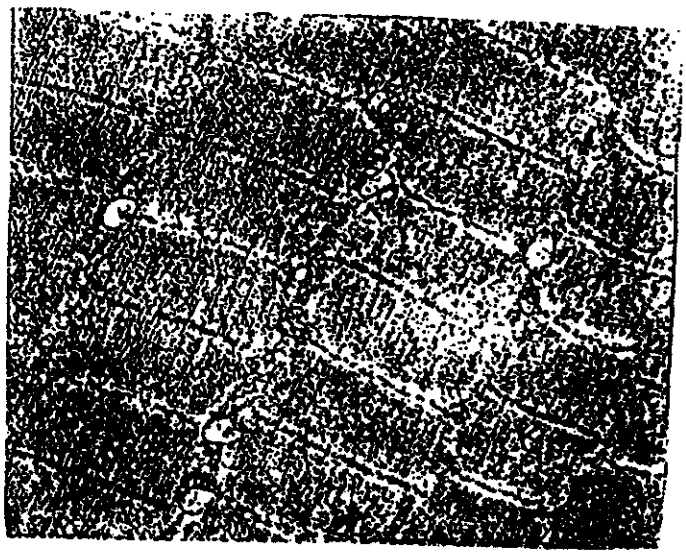


Fig. 26

hake muscle (x=23333)

12 Months.(Bar=0.6µm)

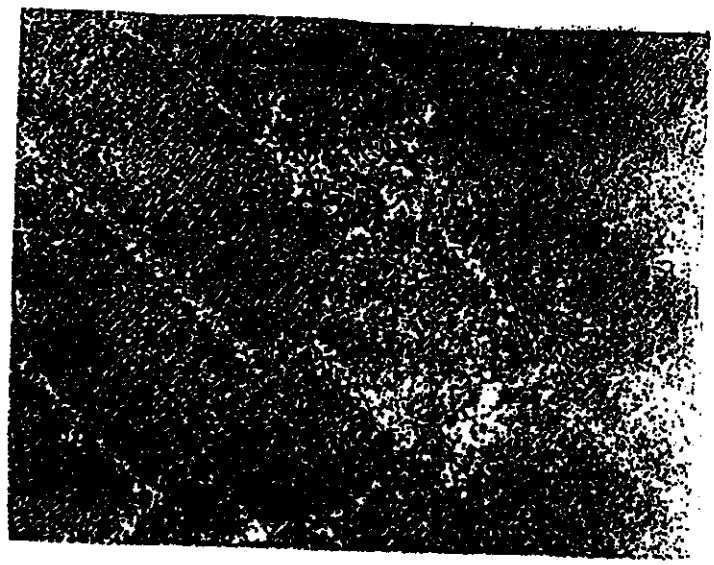


Fig. 27

Sardine muscle (x=40000)

2 Months

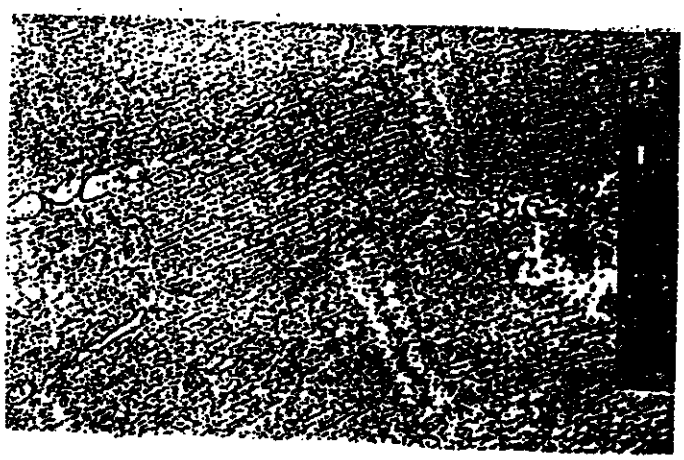


Fig. 28

Sardine muscle (x=40000)

12 Months

Figures from 25 to 28 : The TEM structure of muscles of hake and sardine at 2 and 12 months of storage at -20°C

have been associated with the formation of covalent bonds (Tejada *et al*, 1996). These results were consistent with the electrophoretic profiles of the NaCl-soluble fractions (S1), which confirmed the occurrence of covalent bonding of protein in micro-aggregates as storage progressed.

Lot C: Figs 33 to 35 show supernatants S1 from C minces (50 % hake + 50 % sardine) at 2, 5 and 12 months frozen storage. At 2 months there were few filamentous zones, the protein forming large agglomerations. As storage progressed, aggregated zones also appeared, in which ring-shaped structures were apparent. At 12 months (Fig. 35) most of the proteins appeared to form a tangled network with a less dense structure than in lot B. These results were again borne out by electrophoresis of this fraction.

Lot D: (Figs 36 to 38): At 2 months' storage, all the extracted protein in 0.6M NaCl in lot D (20 % hake + 75 % sardine) appeared as small aggregates which formed globular subunits with no sign of filamentous structures, giving an appearance quite different from the other lots. As storage progressed, protein aggregates containing filaments formed in the supernatant (5 months); however, with the passage of time ring-shaped structures became ever more clearly visible, which was consistent with the increase in covalent aggregates detected by SDS-PAGE.

Lot E: (Fig. 39) : At 2 months' storage, all the extracted protein in 0.6M NaCl in lot E (100 % sardine) appeared as small aggregates similar to the ones observed in lot D. Globular aggregates were apparent from the outset but developed differently from those in the lots with mixtures of hake muscle. As frozen storage progress, the protein in the supernatant adhered to the tube walls leaving no protein to be observed in the supernatant under TEM. This bears out

Fig. 29

Lot A (x = 44000)

2 Months (Bar=0.25 µm)

Fig. 30

Lot B (x = 42500)

2 Months (Bar=0.4 µm)

Fig. 31

Lot B (x = 42500)

5 Months (Bar=0.4 µm)

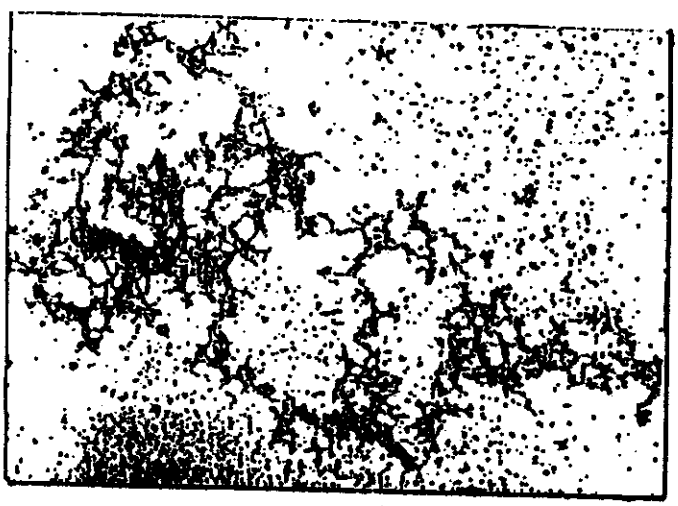


Fig. 32

Lot B (x = 44000)

12 Months (Bar=0.25 µm)

Figures from 29 to 32 : The TEM structure of Supernatant (S1 fractions) of Lot A (100 % minced hake) at 2 monthes and Lot B (75 % minced hake + 25 % minced sardine) at 2, 5 and 12 months of storage at -20°C.

Fig. 33

Lot C (x = 42500)

2 Months (Bar=0.4 µm)

Fig. 34

Lot C (x = 44000)

5 Months (Bar=0.25 µm)

Fig. 35

Lot C (x = 42500)

12 Months (Bar=0.4 µm)

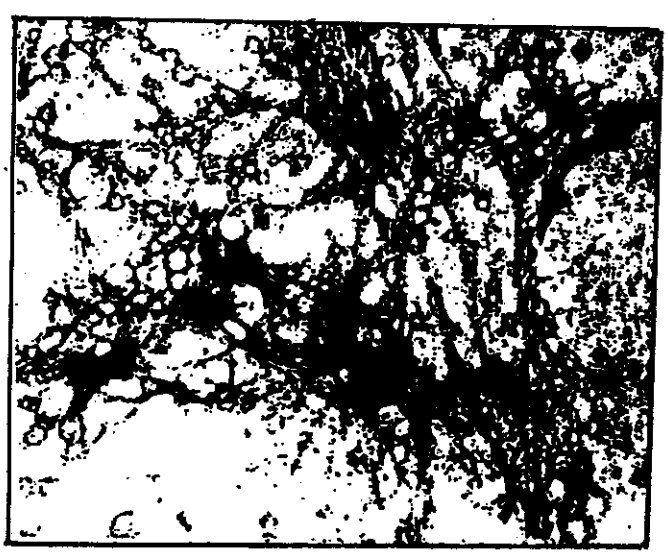


Fig. 36

Lot D (x = 42500)

2 Months (Bar=0.4 µm)

Figures from 33 to 36 : The TEM structure of Supernatant (S1 fractions) of lot C (50 % minced hake + 50 % minced sardine) at 2, 5, and 12 months and lot D (25 % minced hake + 75 % minced sardine at 2 months of storage at -20°C.

Fig. 37

Lot D (x = 44000)

5 Months (Bar=0.25 µm)

Fig. 38

Lot D (x = 42500)

12 Months (Bar=0.4 µm)

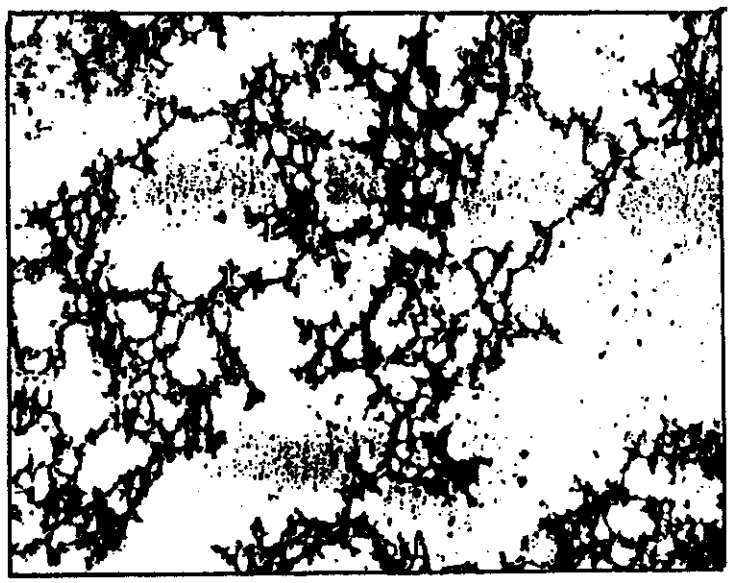


Fig. 39

Lot E (x = 35000)

2 Months (Bar=0.4 µm)

Figures from 37 to 39 : The TEM structure of Supernatant (S1 fractions) of Lot D (25 % minced hake + 75 % minced sardine) at 5 and 12 months and Lot E (100 % minced sardine) at 2 months of storage at -20°C.

observation by SDS-PAGE: the electrophoretic profile of the S1 fractions showed extraction of covalent bond micro-aggregates from the outset that changed very little in this lot. However these micro-aggregates, as frozen storage progress, may form higher structures, that adhere to the tube walls, bond together by secondary or disulfide bonds that break in the electrophoretic conditions.

Mixing of varying percentages of species altered the amount and type of protein extracted in NaCl 0.6M (S1 fractions). In all cases there was increased incidence of aggregates formed by ring-shaped structures, which are thought to be due to the formation of covalent protein-protein bonds. In the mixed-species lots, the amount of NAM extracted and the structure of these S1 fractions differed from those found in the original species. The formation of these aggregates in the mixed-species lots, where there was a progressive increase in soluble NAM aggregates forming covalent bonds as storage progressed, bears out the findings of Careche and Tejada (1990 a), who postulated that the type of interactions involved in hake muscle with added oxidized lipids could be due to covalent bonding rather than secondary forces.

* **Precipitate Morphology (P1 fractions):-**

Photographs 40 to 54 show the precipitates (P1 fractions) obtained after extracting the protein with 0.6M NaCl by the method of Kawashima *et al* (1973) from the different minced lots at 2, 8 and 12 months' storage.

Initially, the muscle structure was generally better retained in the sardine precipitates (Fig 52) and in the lots containing a high percentage of sardine (lot D, Fig 49 and

Fig. 40

Lot A (x = 44000)

2 Months (Bar=0.25 µm)

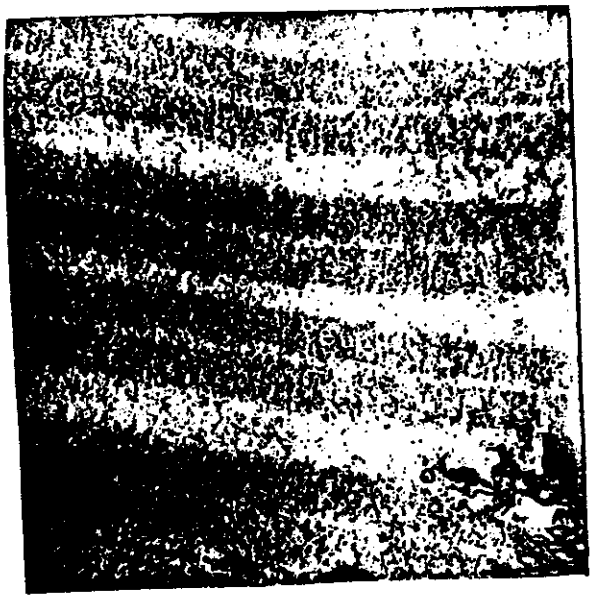


Fig. 41

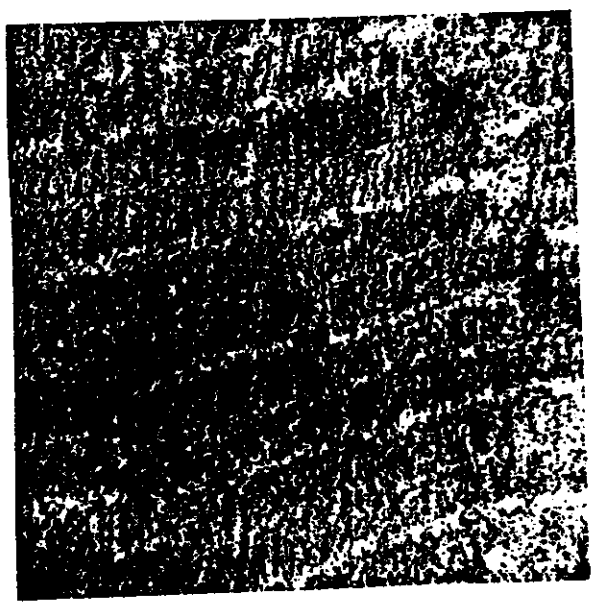
Lot A (x = 44000)

8 Months (Bar=0.25 µm)

Fig. 42

Lot A (x = 44000)

12 Months (Bar=0.25 µm)



Figures from 40 to 42 : The TEM structure of precipitate (P1 fractions) of Lot A (100 % minced hake) at 2, 8 and 12 months of storage at -20°C.

Fig. 43

Lot B (x = 44000)

2 Months (Bar=0.25 µm)

Fig. 44

Lot B (x = 44000)

8 Months (Bar=0.25 µm)

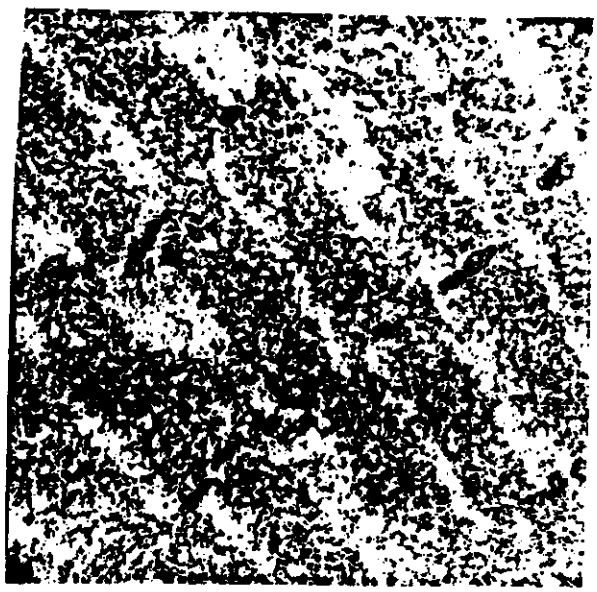
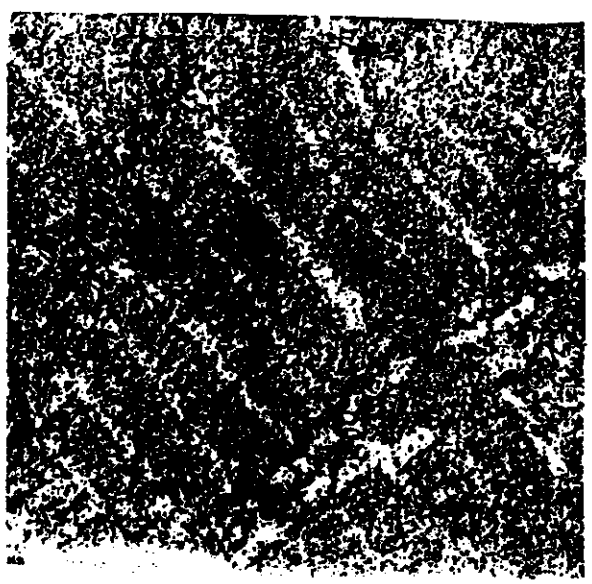


Fig. 45

Lot B (x = 44000)

12 Months (Bar=0.25 µm)



Figures from 43 to 45 : The TEM structure of precipitate (P1 fractions) of Lot B (75 % minced hake + 25 % minced sardine) at 2, 8 and 12 months of storage at -20°C.

Fig. 46

Lot C (x = 44000)

2 Months (Bar=0.25 µm)

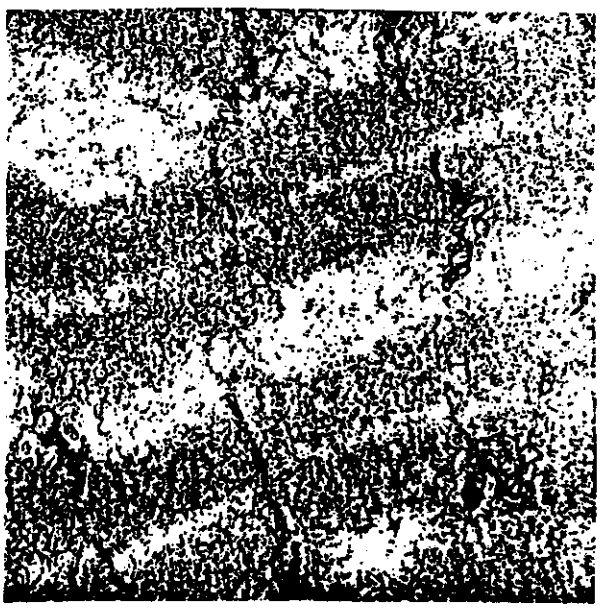


Fig. 47

Lot C (x = 44000)

8 Months (Bar=0.25 µm)

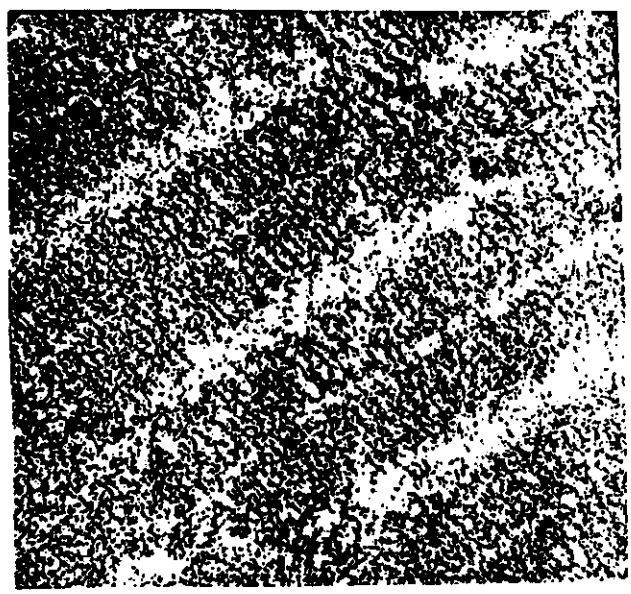


Fig. 48

Lot C (x = 44000)

12 Months (Bar=0.25 µm)

Figures from 46 to 48 : The TEM structure of precipitate (P1 fractions) of Lot C (50 % minced hake + 50 % minced sardine) at 2, 8 and 12 months of storage at -20°C.

Fig. 49

Lot D (x = 44000)

2 Months (Bar=0.25 µm)

Fig. 50

Lot D (x = 44000)

8 Months (Bar=0.25 µm)

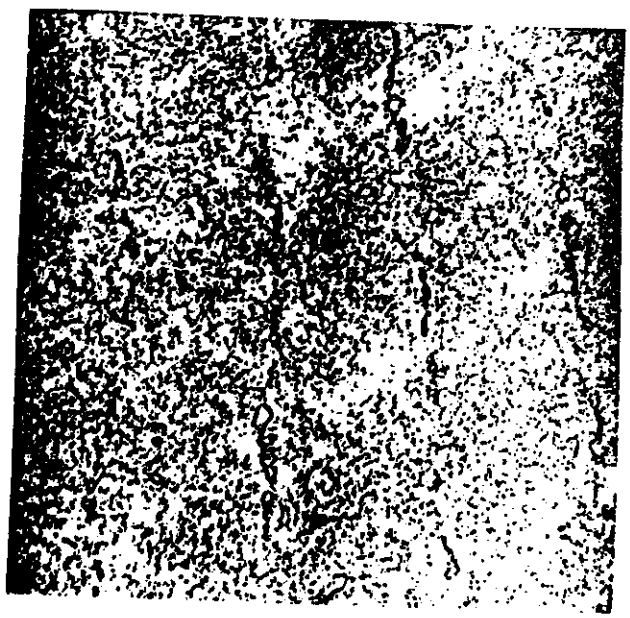
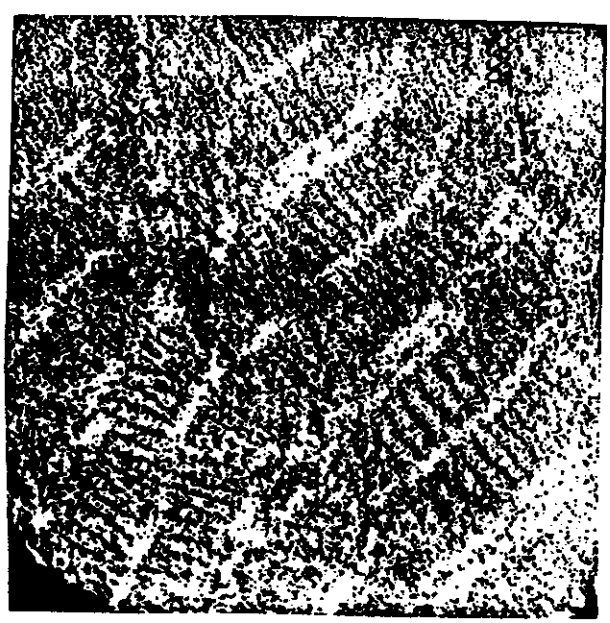


Fig. 51

Lot D (x = 44000)

12 Months (Bar=0.25 µm)



Figures from 49 to 51 : The TEM structure of precipitate (P1 fractions) of Lot D (25 % minced hake + 75 % minced sardine) at 2, 8 and 12 months of storage at -20°C.

Fig. 52

Lot E (x = 44000)

2 Months (Bar=0.25 µm)

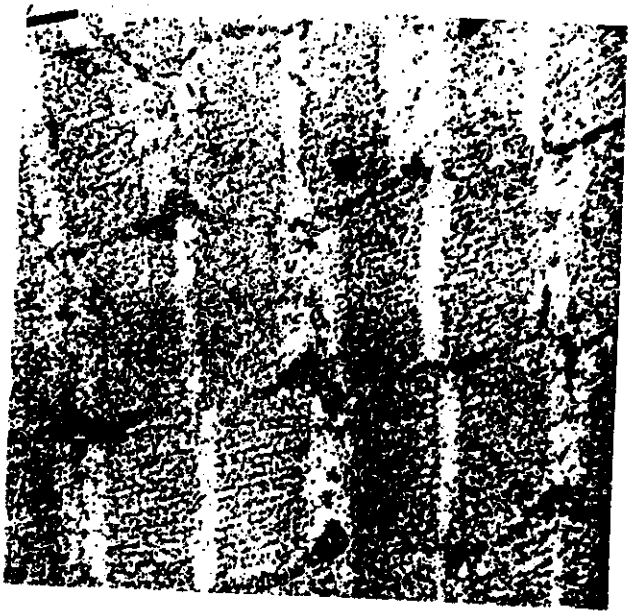


Fig. 53

Lot E (x = 44000)

8 Months (Bar=0.25 µm)

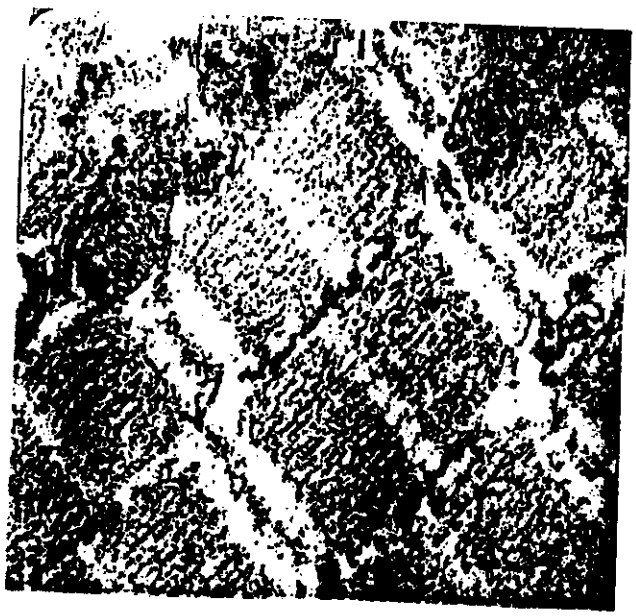
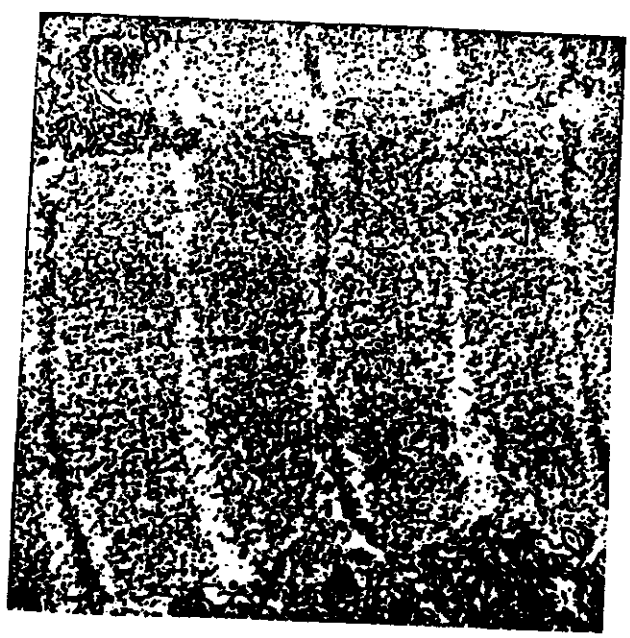


Fig. 54

Lot E (x = 44000)

12 Months (Bar=0.25 µm)



Figures from 52 to 54 : The TEM structure of precipitate (P1 fractions) of Lot E (100 % minced sardine) at 2, 8 and 12 months of storage at -20°C.

lot C, Fig 46), and less so where the percentage of hake was higher (lot B, Fig 43). In the hake lot (lot A, Fig 40), scarcely any of line Z was visible.

As storage progressed (8 months), the insoluble P1 aggregates exhibited denser structures in parallel alignment, which could be associated with the formation of aggregates among the actin and myosin myofilaments (Figs 41, 44, 47, 50 and 53).

By the end of storage (12 months), the structure in the P1 insoluble aggregates from hake (Fig 42) was more organized than at the outset (Fig 40) and traces of line Z were visible. The dense structures detected at 8 months were also apparent. In the sardine lots on the other hand, at 12 months (Fig 54) the structure displayed a clearly defined Z line and was very similar to that of the original sarcomere (Fig 28), although the structures appeared more disorganized after extraction. The mixed-species lots exhibited structures half-way between the hake and the sardine lots (Figs 45, 48 and 51).

The appearance of dense structures was consistent with the increase of covalent aggregates detected in the S2 fractions by SDS-PAGE.